Transcriptional Regulation of the Gonadotropin-Releasing Hormone Receptor (GnRHR) gene by Glucocorticoids.

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Thesis submitted in fulfilment of the requirements for the Degree of Master of Science (Biochemistry) at the University of Stellenbosch

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own, original work (unless acknowledged otherwise) and that neither the whole nor any part of it has been, is being or is to be submitted for another degree at this or any other University.

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Date

Abstract

The gonadotropin-releasing hormone (GnRH) receptor is a G-protein-coupled receptor in the pituitary gonadotropes and is an important control point for reproduction. GnRH binds to the GnRH receptor (GnRHR) resulting in the synthesis and release of follicle stimulating hormone (FSH) and luteinizing hormone (LH). The sensitivity of the pituitary to GnRH can be directly correlated with GnRHR levels. The mouse GnRHR promoter contains three *cis* elements containing binding sites for steroidogenic factor-1 (SF-1), namely site 1 (-15/-7), site 2 (-244/-236) and site 3 (-304/-296) as well as an activator protein-1 (AP-1)-like consensus sequence (TGAGTCA) at position –336/-330. While sites 1 and 2 and the AP-1 site have been previously shown to be involved in regulation of transcription of the mouse GnRHR (mGnRHR) promoter in some cell lines, the role of site 3 has not been previously investigated. This study investigated whether transcription of the mGnRHR gene is regulated by GnRH and glucocorticoids in the LβT2 gonadotrope pituitary cell line, and the role therein of site 3 and the AP-1 site and their cognate proteins, using a combination of *in vitro* protein-DNA binding studies and promoter-reporter assays. The role played by site 3 and the AP-1 site in basal transcription of the mGnRHR gene in LβT2 cells was the first area of investigation during this study. Luciferase reporter plasmids containing 600 bp of the mGnRHR promoter were used where the site 3 and AP-1 sites were either wild-type or mutated. Two constructs were prepared from the wild-type construct, i.e. wild type (LG), site 3 mutant (m3) and AP-1 mutant (mAP-1). Transfection of LG, m3 and mAP-1 plasmids into LβT2 cells was carried out to determine the effect of these mutations on the basal expression of the mGnRHR gene. Mutation of site 3 resulted in a 1.5 fold increase in the transcriptional activity of the mGnRHR promoter. This suggests that site 3 plays a role in the inhibition of basal transcriptional levels of the mGnRHR promoter in LβT2 cells. Mutation of the AP-1 site resulted in a 50% decrease in basal transcriptional levels of the mGnRHR promoter in LβT2 cells. This suggests that the AP-1 site is involved in positively mediating the basal transcriptional response of the GnRHR promoter in LβT2 cells. Experiments towards the understanding of the mechanism of the *cis* elements (site 3 and AP-1 site) on the mGnRHR promoter were carried out along with the role of protein kinase A (PKA) pathways, proteins involved and the effect of varying doses for varying times of GnRH, as well as the over-expression of PKA and the SF-1 protein. It was found that site 3 and the AP-1 site are not involved in the GnRH response. Results suggest that site 3 is partially involved in the PKA response in LβT2 cells. Site 3 can bind SF-1 protein as shown via competitive electrophoretic mobility shift assays (EMSA). When EMSA's were performed on the AP-1 site the findings were that the c-Fos protein was not involved in the activation of the AP-1 site. A factor was found to bind to the AP-1 site, which did not require the intact AP-1 site, suggesting that it could be the c-Jun protein that binds to the AP-1 site under basal conditions.

Another area that was investigated was whether the mGnRHR promoter can be regulated by dexamethasone (dex) either via the AP-1 site or site 3. A dose and time-dependent increase

in promoter activity was observed with dex. This effect appears to require site 3 and the AP-1 site, as shown by the complete loss of response when these sites were individually mutated, consistent with a functional interaction between site 3 and the AP-1 site in LβT2 cells.

Opsomming

Die gonadotropienvrystellings hormoon (GnRH) reseptor is ‘n G-proteïen-gekoppelde reseptor in die pituitêre gonadotrope en is ’n belangrike beheerpunt vir reproduksie. GnRH bind aan die GnRH reseptor (GnRHR) met die gevolg dat follikel stimulerende hormoon (FSH) en luteïeniserende (LH) gesintetiseer en vrygestel word. Die sensitiwiteit van die pituitêre klier vir GnRH kan direk met GnRHR vlakke gekorreleer word. Die muis GnRHR promotor bevat drie *cis* elemente met bindingssetels vir steroïedogeniese faktor 1 (SF1), naamlik setel 1 (-15/-7), setel 2 (-244/-236) en setel 3 (-304/-296) sowel as ’n aktiveerder proteïen 1 (AP-1) tipe konsensus sekwens (TGAGTCA) in posisie -336/-330. Terwyl setels 1 en 2 en die AP-1 setel voorheen getoon is om by die regulering van transkripsie van die muis GnRHR (mGnRHR) promotor in party sellyne betrokke te wees, is die rol van setel 3 nog nie vantevore bestudeer nie. In hierdie studie is ondersoek of die transkripsie van die mGnRHR geen deur GnRH en glukokortikoïede in die LβT2 gonadotroop pituitêre sellyn gereguleer word, en die rol van setel 3 en die AP-1 setel en hulle binders, deur gebruik te maak van *in vitro* proteïen-DNA bindings studies en promotor-verslaggewer essais. Die rol wat setel 3 en die AP-1 setel in basale transkripsie van die mGnRHR gene in LβT2 selle gespeel het, was die eerste onderwerp wat in hierdie studie bestudeer is. Lusiferase verslaggewer plasmiede wat die eerste 600 bp van die mGnRHR promotor bevat het en waarin setel 3 en die AP-1 setels óf wilde tipe óf gemuteer was, is gebruik. Two konstrukte is vanaf die wilde tipe konstruk berei, naamlik wilde tipe (LG), ’n setel 3 mutant (m3) en ’n AP-1 mutant (mAP-1). Transfeksie van LG, m3 en mAP-1 plasmiede in LβT2 selle is deurgevoer om te bepaal wat die effek van hierdie mutasies op die basale ekspressie van die mGnRHR gene was. Mutasie van setel 3 het ’n 1.5-voudige toename in die transkripsionele aktiwiteit van die mGnRHR promotor tot gevolg gehad. Dit suggereer dat setel 3 ’n rol in die inhibisie van die basale transkripsievlakke van die mGnRHR promotor in LβT2 selle speel. Mutasie van die AP-1 setel het tot ‘n 50% verlaging in basale transkripsievlakke van die mGnRHR promotor in LβT2 selle gelei. Dit suggereer dat die AP-1 setel betrokke is in die positiewe bemiddeling van die basale transkriptionele respons van die GnRHR promotor in LβT2 selle. Eksperimente wat gemik was om die meganisme van die *cis*-elemente (setel 3 en die AP-1 setel) op die mGnRHR promotor te verklaar, asook om die rol van proteïen kinase A (PKA) paaie, proteïene daarby betrokke en die effek van varieende dosisse vir verskillende tye van GnRH, sowel as die oorekspressie van PKA en die SF-1 proteïen, is deurgevoer. Dit is gevind dat setel 3 en die AP-1 setel nie betrokke by die GnRH respons is nie. Die resultate suggereer dat setel 3 gedeeltelik betrokke is by die PKA respons van LβT2 selle. Setel 3 kan SF-1 proteïen bind soos getoon deur kompeterence elektroforetiese mobiliteits verskuiwings essais (EMSA). As EMSA’s deurgevoer is op die AP-1 setel is bevind dat die c-Fos proteïen nie betrokke is in die aktivering van die AP-1 setel nie. ’n Faktor is gevind om aan die AP-1 setel te bind wat nie ’n intakte AP-1 setel vereis het nie, wat gesuggereer het dat dit die c-Jun proteïen kan wees wat aan die AP-1 setel onder basale omstandighede bind.

’n Ander area wat ondersoek is, is of die GnRHR promotor gereguleer kan word deur deksametasoon (dex) óf via die AP-1 setel óf via setel 3. ’n Dosis en tyds-afhanklike toename in promotor aktiwiteit is waargeneem met dex. ’n Vereiste vir hierdie effek blyk om die teenwoordigheid van setel 3 en die AP-1 setel te wees, soos aangetoon deur die totale verlies aan response as hierdie twee setels individueel gemuteer is, en wat weereens in ooreenstemming met die funksionele interaksie tussen setel 3 en die AP-1 setel in LβT2 selle is.

Acknowledgements

I would like to thank the following people:

- My supervisor, Professor Janet Hapgood, for all her support, understanding and guidance.
- Hanel Sadie for guidance and encouragement and mentoring of experiments.
- Carmen Langeveldt for tissue culture cells and support.
- Donita Africander and Elizabeth Stubsrud for personal support and encouragement.
- Dr Amanda Swart for helping of literature searches.
- Zyno Allie for guidance and understanding.
- My fellow postgraduate biochemistry students.
- Big thanks to Mathew Connelly for help with the computer support.
- The National Research Foundation for financial support.
- To my family and William Gillespie for encouragement, love, support and understanding.

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Chapter 1: Introduction

1.1. Gonadotropin-Releasing Hormone and its Receptor: In the Hypothalamic-Pituitary-Gonadal axis

1.1.1 Characterization of the GnRH-Receptor.

The GnRHR protein has seven putative membrane-spanning domains and encodes a 327- to 328-amino acid protein. The lack of a cytoplasmic carboxyl terminal tail makes the GnRHR a unique protein among the G protein-coupled receptors.

The GnRHR promoter contains special features such as 1) the aspartic acid (D), arginine (R) and tyrosine (Y) (DRY) to aspartic acid (D), arginine (R) and serine (S) (DRS) consensus sequence, which is present on the second intra-cytoplasmic loop, 2) On the second and seventh intra-membrane domains there is an interchange of the conserved D and N residues and 3) it lacks the typical intracellular carboxyl terminus making it one of the smallest receptors that contain the seven transmembrane structure [1, 2].

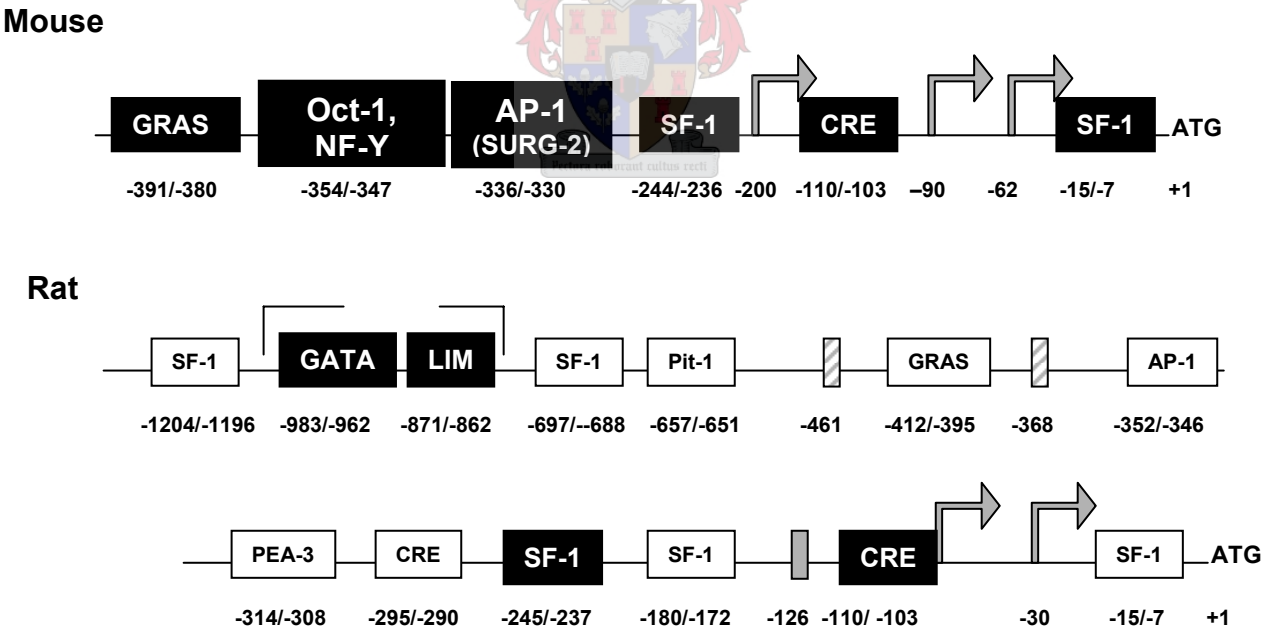


Figure 1: The functional elements in the GnRHR I promoter regions of the mouse and rat genes. The following boxes on the diagram above are represented as follows: the shaded boxes and striped boxes are the TATA and CCAAT elements respectively. Black boxes represent elements, which have been functionally characterized. Putative elements shown as white boxes have been identified through

Figure 1 legend continuation:
promoter sequence analysis. The arrows indicate the transcriptional start sites, and the translational start site is represented as "ATG". Abbreviations: SF-1 = Steroidogenic Factor-1 binding site; Pit-1 = Pit-1 transcription factor binding site; CRE = cAMP response element; AP-1 = activator protein 1 binding site; PEA-3 = phorbol ester response element; Oct-1 = octamer transcription factor-1 binding site; LIM = LIM-homeodomain factor binding site; GRAS = GnRH receptor activating sequence; SURG = sequence underlying responsiveness to GnRH, NF-Y = nuclear factor-Y binding site. Taken from Hapgood *et al. (*2005) [3].

GnRHR genes that have been cloned and characterized include those from human, rat, mouse and pig [2, 4] and many others. The genes in these species exist as single copies and have a high degree of sequence homology in their coding regions.

The GnRHR gene contains three exons that are separated by two introns and is located on chromosome 5 in the mouse, chromosome 4q13.2-21.1 in humans and on chromosome 6 in sheep. The size of the introns as well as the sequence and length of the 5‘ and 3‘ untranslated regions differ dramatically between the different species even though the exon-intron boundaries are conserved between the species [2]. Alternative splicing of the RNA transcripts can occur in the mouse and it results in multiple GnRHR mRNAs that code for truncated proteins. In the human and rat multiple GnRHR RNA transcripts have been discovered in tissues other than the anterior pituitary such as the ovaries, testes, brain, prostrate, breast and placenta [2].

1.1.2 Characterization of the Gonadotropin Releasing Hormone (GnRH)

The integration and precise orchestration of hormonal regulation at the hypothalamic, pituitary, and gonadal levels is necessary for the regulation of normal mammalian sexual maturation and reproductive function [1] and pregnancy in adults [5, 6, 7]. GnRH is the key endocrine hormone, which regulates reproduction.

GnRH is a decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2)[8], (see figure 2) which is released in synchronized pulses into the hypophyseal portal vascular system every 30-120 minutes [8].

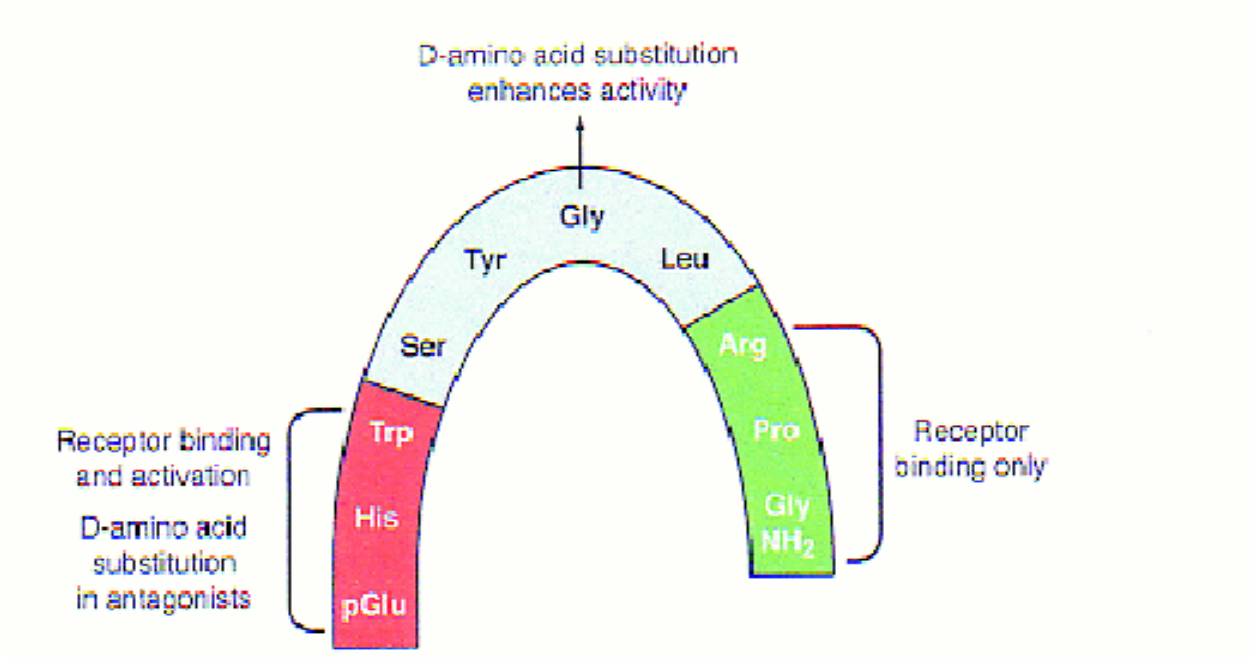


Figure 2: A schematic representation of the mammalian GnRH. GnRH is in its folded conformation in which it is bound to the GnRH pituitary receptor. This figure was taken from Millar *et al.* (2004) [8].

GnRH is delivered to the anterior pituitary where it binds specifically with high affinity to the G-protein coupled receptor (GPCR) on the pituitary gonadotropes called the GnRH receptor (GnRHR) [9]. Of all the cells in the anterior pituitary gland, the gonadotropes only make up 8-15 % [10]. GnRH responsiveness depends on the number of GnRHR's on the cell surface [11]. There is evidence to suggest that the GnRHR numbers are partially dependent upon the level of GnRHR mRNA in gonadotropes [11]. The partial correlation of GnRHR numbers with the changes in steady state GnRHR mRNA levels has been shown to be partly regulated at the transcriptional level [11].

When GnRH was first isolated from the hypothalamus of mammals it was thought to be a unique peptide with its sole function being to regulate luteinizing hormone (LH) and follicular stimulating hormone (FSH) release from the pituitary, within the hypothalamus-pituitary-gonadal (HPG) axis (mentioned later). It later became apparent that diverse forms exist in vertebrates [8], which has subsequently led to the identification of 23 different forms of GnRH (see figure 3). For many years GnRH and its analogs have been used in the treatment of hormone-dependent diseases, as well as for assisted reproductive techniques [8]. There is also potential to use GnRH and its analogs as a male and female contraceptive [8]. The stimulation of endogenous GnRHR levels by low doses of synthetic GnRH (in a pulsatile fashion) has also been shown to restore fertility in hypogonadal men and women [8]. It has also been said to be an effective treatment for un-descended testes and delayed puberty [8].

	1	2	3	4	5	6	7	8	9	10
Mammal	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly	NH_2
Guinea pig	pGlu	Tyr	Trp	Ser	Tyr	Gly	Val	Arg	Pro	Gly	NH_2
Chicken 1	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Gln	Pro	Gly	NH_2
Rana d.	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Trp	Pro	Gly	NH_2
Seebream	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Ser	Pro	Gly	NH_2
Salmon	pGlu	His	Trp	Ser	Tyr	Gly	Trp	Leu	Pro	Gly	NH_2
Medaka	pGlu	His	Trp	Ser	Phe	Gly	Leu	Ser	Pro	Gly	NH_2
Catfish	pGlu	His	Trp	Ser	His	Gly	Leu	Asn	Pro	Gly	NH_2
Herring	pGlu	His	Trp	Ser	His	Gly	Leu	Ser	Pro	Gly	NH_2
Dogfish	pGlu	His	Trp	Ser	His	Gly	Trp	Leu	Pro	Gly	NH_2
Chicken II	pGlu	His	Trp	Ser	His	Gly	Trp	Tyr	Pro	Gly	NH_2
Lamprey III	pGlu	His	Trp	Ser	His	Asp	Trp	Lys	Pro	Gly	NH_2
Lamprey I	pGlu	His	Tyr	Ser	Leu	Glu	Trp	Lys	Pro	Gly	NH_2
Chelyosoma I	pGlu	His	Trp	Ser	Asp	Tyr	Phe	Lys	Pro	Gly	NH_2
Chelyosoma III	pGlu	His	Trp	Ser	Leu	Cys	His	Ala	Pro	Gly	NH_2
Ciona I	pGlu	His	Trp	Ser	Tyr	Ala	Leu	Ser	Pro	Gly	NH_2
Ciona II	pGlu	His	Trp	Ser	Leu	Ala	Leu	Ser	Pro	Gly	NH_2
Ciona III	pGlu	His	Trp	Ser	Asn	Gln	Leu	Thr	Pro	Gly	NH_2
Ciona IV	pGlu	His	Trp	Ser	Tyr	Glu	Phe	Met	Pro	Gly	NH_2
Ciona V	pGlu	His	Trp	Ser	Tyr	Glu	Tyr	Met	Pro	Gly	NH_2
Ciona VI	pGlu	His	Trp	Ser	Lys	Gly	Tyr	Ser	Pro	Gly	NH_2
Ciona VII	pGlu	His	Trp	Ser	Asn	Lys	Leu	Ala	Pro	Gly	NH_2
Octapus	pGlu	His	Trp	His	Phe	Ser	Trp	His	Pro	Gly	NH_2

Asn Gly

Figure 3: Primary amino acid sequence of structural GnRH variants spanning approximately 600 million year of evolution. Areas within the left and right box are the conserved NH_2 and COOH-terminal residues that play important functional roles. Figure taken from Millar *et al.* (2004) [8].

Expression of the gonadotropin subunit genes LH and FSH as well as their synthesis and release is regulated by the binding of GnRH to the GnRHR [9] (see figure 4). When a pulse of GnRH is delivered to the anterior pituitary a pulse of LH is released, as LH tends to be stored and is largely dependent on GnRH for secretion. A FSH pulse is also released although it is less distinct as FSH is constitutively secreted and more dependent on biosynthesis for its secretion [8]. Ovulation, corpus luteum formation (in females) and androgen secretion (in males) is regulated by LH [1], whereas growth and maturation of the ovarian follicles (in females) and spermatogenesis (in males) is regulated by FSH [1].

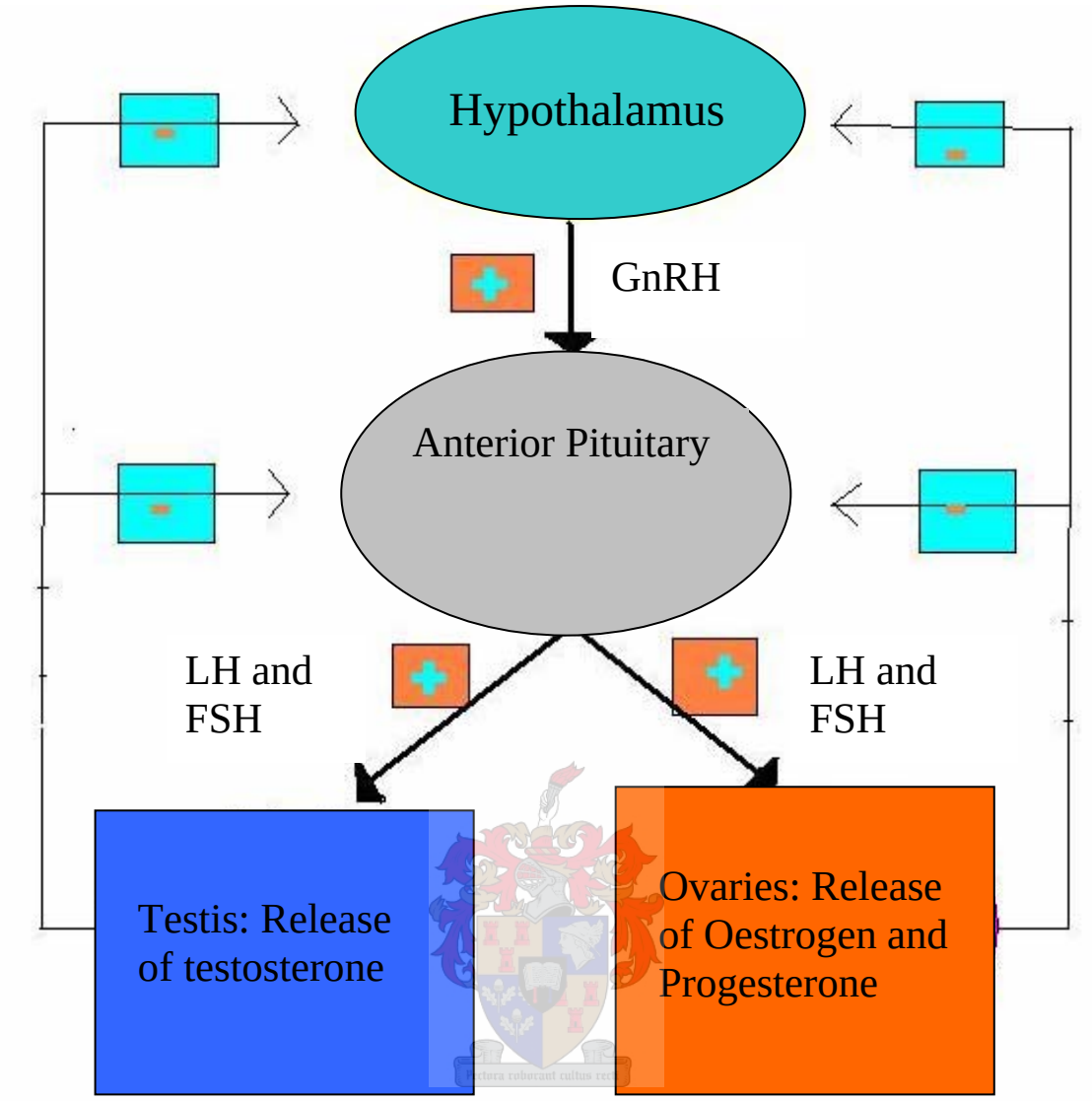


Figure 4: The Hypothalamus-Pituitary-Gonadal (HPG) axis. GnRH is released from the hypothalamus to the anterior pituitary where it binds to GnRHR and releases LH and FSH. It then stimulates the production of testosterone in males and oestrogen and progesterone in females. The HPG axis has a negative feedback cycle. When testosterone, FSH and LH increases to over the threshold the production of GnRH in the hypothalamus is stopped. Positive signs indicate the positive loop system and negative signs indicate the negative loop system. Diagram by Fernandes (2006).

A change in GnRH pulse frequencies causes an asynchronous pattern of LH and FSH release, which results in the modulating effects and the differences in the half-lives of these two hormones [8]. The frequency and amplitude of GnRH regulates the GnRHR [12].

1.1.3 GnRHR promoter

The first proximal promoter that was isolated and characterized was the mouse GnRHR promoter [12]. 1.2kb of 5' flanking region of the mouse GnRHR gene (designated -1164/+62 relative to the major transcriptional start site) has been shown to contain tissue-specific promoter activity [2]. At 62 nucleotides upstream of the translational start site a major transcriptional start site was identified, which appears not to use a TATA box (in αT3-1 cells) [1] although there may be other elements that are important for the accurate start site for transcription such as the GATA motif at position -30 [2].

On the mouse GnRHR promoter (in αT3-1 cells) gonadotrope-specific activity has been reported to be mediated via a tripartite basal enhancer consisting of a steroidogenic factor-1 (SF-1) binding site at –244/-236 [13] a consensus activator protein-1 (AP-1) site at –336/-380 and a GnRHR-activating sequence (GRAS) at –391/-380 [14] (see figure 1).

The transcriptional start site in αT3-1 cells of the rat proximal GnRHR I promoter was first reported to be at 103bp upstream from the start codon containing a putative TATA box 23bp upstream from the transcriptional start site [15]. Later the group of Pincas *et al.(1998)* [16] discovered that there was multiple transcriptional start sites in αT3-1 cells consisting of four clustered around –103 and one situated at –30 together with several minor start sites [16] (See figure 1). The rat GnRHR promoter cooperates with a distal enhancer element called GnRHR-specific enhancer (GnSE), which lies between –1135 and –753 and facilitates gonadotrope-specific expression [16]. For full functionality, the distal enhancer can only function in the context of the proximal promoter and requires the SF-1 site at –245. Therefore, in the mouse and rat GnRHR promoters one can clearly see that the mechanisms involved in gonadotrope-specific expression are different between the mouse and rat GnRHR promoters.

1.2 Regulation of the GnRHR

Several different kinase proteins which target various transcription factors are important in the regulation of the GnRHR promoter. These key "players" include protein kinase A (PKA), protein kinase C (PKC), Steroidogenic factor-1 (SF-1) and the activator protein-1 (AP-1).

1.2.1 Protein Kinase A (PKA) and Protein Kinase C (PKC) in the signal transduction pathway of the GnRHR gene.

The regulation of the GnRHR gene by GnRH occurs via two major signalling transduction pathways namely PKA or PKC. There are many suggestions that different cell lines lead to the use of different signalling pathways. For instance, Maya-Nunez *et al.* (1999) [17] found in GGH_3 cells that the major signalling pathway involved in GnRH-dependent GnRH receptor gene regulation in the rat was the PKA pathway. Norwitz *et al.* (1999) [2] found that in αT3-1 cells the major signalling pathway that causes a response to GnRH in the mouse and the human GnRH receptor gene is through the PKC pathway and not via the PKA pathway, which was in contrast to what Maya Nunez *et al.* (1999) [17] found in GGH_3 cells. White *et al.* (1999) [18] found that the activation of the GnRHR promoter by GnRH in αT3-1 cells was dependent on the AP-1 binding site and was found to mediate via the PKC pathway. Even if the

response of the GnRHR promoter to GnRH in αT3-1 cells is regulated via the PKC pathway, it is possible that the PKA pathway can also regulate the gene in response to other signalling molecules that activate the PKA pathway.

1.2.2 Characterization of Steroidogenic Factor-1 (SF-1).

SF-1 protein is an important regulator of the mGnRHR promoter and more background will be given on the SF-1 transcription factor since it has been shown to be involved in mGnRHR transcriptional regulation. SF-1 was discovered in the early 1990s and was found to be a key regulator of steroidogenic genes involved in steroidogenesis [19]. The 53kDa protein was also called Ad4BP (Adrenal 4 Binding-Protein), because it was identified as the protein that bound to the Ad4 cis-element of the bovine cytochrome P-450 gene (CYP11B) [20], and formally known as NR5A1 (nuclear receptor subfamily 5, group A, member 1) [21]. The SF-1 protein is expressed in steroidogenic tissues and has also been identified in other tissues [21]. The cDNA of SF-1 was successfully cloned by two teams Morohashi *et al*. (1992) [22] and Honda *et al*. (1993) [20]. Both of these teams identified a 53kDa protein and a 1383bp cDNA that encoded for a protein of 461 amino acids [20, 22]. Through sequence homology researchers found that mouse SF-1 and Drosophila Ftz-F1 had a high degree of homology to each other. The gene encoding SF-1 was called Ftz-f1 in Drosophila. Ftz-F1 transcription factor controls fushi tarazu homeotic gene expression [19]. Four different proteins are encoded for by this gene namely ELP1, ELP2, ELP3 and SF-1, which is produced by using alternative promoters and splicing. There has been some suggestion that embryonal long terminal repeat-binding protein (ELP) in Drosophila plays a role in regulating transcription of nuclear receptors. SF-1 on the other hand has been shown to be the key activator of steroidogenic endocrine function in mammals [19].

SF-1 is a member of the orphan nuclear receptor superfamily, which includes receptors for steroid, thyroid, and retinoid hormones [21]. The reason SF-1 is called an orphan nuclear receptor is because it does not have a *bona fide* SF-1 ligand. These orphan receptors have conserved functional domains that include two zinc fingers, which mediate its binding to DNA, a proline rich domain, which has been proposed to mediate transcriptional activation, and a AF-2 transactivation domain [19].

The first zinc finger of SF-1 contains a proximal (P) box and the second zinc finger contains a distal (D) box and between these two zinc fingers is an intervening linker region that seems to be conserved among human, bovine, rat and mouse [23] (see figure 5). The P box for the DNA sequence half sites of the hormone responsive elements determines the recognition, whereas the D box determines the appropriate spacing of these half sites. Adjacent to the D box is another element, which recognizes additional bases termed the A-box.

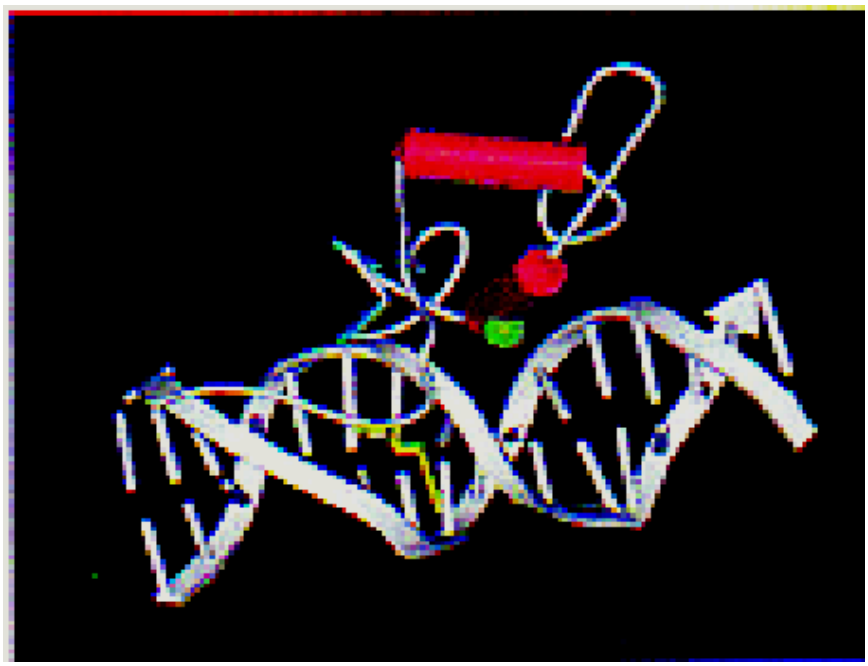


Figure 5: Regulation of transcription by SF-1. The P-Box is shown in green whereas the A-Box is shown in yellow. Taken from Achermann *et al.,* (2002) [24].

SF-1 was found to be expressed in the embryonic forebrain, hypothalamus and the pituitary. Researchers found that in SF-1 knockout mice the expected birth rate frequency was 25% indicating that SF-1 was not required for embryonic survival [25]. However these researchers did find that mice that had the SF-1 knockout had external female genitalia, irrespective of genetic sex and also had failure to produce testicular androgens and that their survival rate after birth was poor, which was due to a corticosteroid deficiency, indicating the important role that SF-1 plays in androgen and corticosteroid biosynthesis [25]. In these SF-1 knockout mice adrenal glands and gonads were completely absent revealing the importance of SF-1 in the development of these tissues [25]. Another study with SF-1 knockout mice showed a lack of the ventromedial hypothalamic nucleus, which is generally rich in steroid hormone receptors and has been shown to be involved in female reproductive behaviour [25]. The spleen of mice that contained the SF-1 knockout gene showed severe developmental defects. Therefore, from these different studies it is clear that SF-1 is a key regulator at multiple levels of the hypothalamus-pituitary- adrenal (HPA)/HPG axis (explained in more detail later).

1.2.3 Binding of SF-1 to DNA

It is well established that most nuclear receptors bind to DNA in one of two ways either by direct or inverted dimerization. However, SF-1 binds as a monomer to DNA. SF-1 DNA-binding sites have been identified and shown to be functional in several promoters of genes involved in steroidogenesis. The activation of transcription is caused by the SF-1 protein binding to the SF-1 site on the promoter resulting in the shuttling of nuclear receptors from the cytoplasm, where they bind to their cognate ligands, to the nucleus. Nuclear localization signals (NLS) are important for the shuttling of nuclear receptors. SF-1 contains one NLS downstream of the DNA binding domain (DBD) (amino acids 89-

101), and is important for transcriptional activity [19]. In the ligand-binding domain (LBD) of SF-1, helices 1 and 12 are able to adopt an active confirmation independently of a ligand. This occurs due to the phosphorylation of a serine residue at position 203. SF-1 also contains a conserved AF-2 domain, which is responsible for recruiting co-activators. The AF-2 domain of SF-1 co-operates with two activating domains, which are present downstream from the DBD. The two domains are the proximal activation domain and helix H1. The proximal activation domain overlaps with the hinge area and helix H1 is present in the putative LBD (amino acid 187-245). These domains are important for maximal SF-1 activity together with the co-activator SRC-1 [26].

SF-1 is said to be a master protein directing its effects at multiple levels of the reproductive axis, as it is present at multiple levels of the HPG axis [26]. The SF-1 site present at position -250 to -232 is part of the tripartite enhancer, which confers gonadotrope-specific expression in murine GnRHR gene. The mutation of this site resulted in a 58% decrease in promoter activity [14]. On the human GnRHR promoter the SF-1 site is situated at -140/-134 and has been shown to be involved in mediating high cell-specific expression in αT3-1 cells [27]. This high cell-specific expression however has not been assigned for similar sites in the mouse and rat GnRHR promoters (position -15/-7).

1.2.4 Regulation of SF-1 expression

SF-1 is involved in mediating the PKA response in certain genes. What this means is that the SF-1 protein is required for the PKA pathway-dependent increase in transcription in certain genes.

There are two mechanisms thought to be involved in regulating SF-1, which are

1) SF-1 transcriptional activity can be affected by it being a direct target for PKA-dependent phosphorylation resulting in the SF-1 protein binding to the target promoter or,

2) The levels of the SF-1 protein can be affected by PKA, via a PKA dependent regulation of the SF-1 protein, where the target for PKA is the transcription factor that regulates the expression of the SF-1 gene (i.e. indirect mechanism).

The indirect effect was investigated by Shapiro *et al*. (1996) [28] and found that when rat granulosa cells were incubated with 8-Br-cAMP there was a 2-fold increase in SF-1 mRNA. Therefore, they suggested that this increase in SF-1 mRNA could contribute to the increase in transcription of the GnRHR gene. Since the increase in SF-1 mRNA appeared to be insufficient to explain the full increase in GnRHR activity, the authors suggested that SF-1 protein could also be modified post-translationally to increase its activity in response to the stimulation of the PKA pathway [28]. Zhang *et al.* (1996) [29] showed that when they performed transfection studies on mouse Leydig MA-10 and adrenocortical Y-1 cells that the SF-1 protein can bind to an element in the promoter of the cytochrome P450 17α-Hydroxylase/c17-20 lyase gene and that SF-1 mediates the stimulatory effect of the PKA pathway on transcriptional activity [29].

There are other binding sites on the rat aromatase gene promoter that are required for transcriptional activation by PKA e.g. cAMP response element (CRE)[30]. The binding sites required for transcriptional activation via PKA are different for different promoters. For instance in the human aromatase gene promoter there appears to be no classical CRE elements present and it is thought that the PKA-stimulated transcription is due to increased levels and DNA-binding of SF-1 [31]. In conclusion the exact mechanism whereby SF-1 mediates the PKA response differs from gene to gene as well between the same gene in different species.

1.3 Different model systems

There are several model systems, which are currently being used in order to study the regulation of the mouse GnRHR gene. The most commonly used model systems are transformed cell lines, which originated from specific tissue types. Cell lines tend to be mostly derived from tumors, which are induced in transgenic mice. Below are some common cell lines used to study the regulation of the mouse GnRHR gene in the pituitary:

1.3.1 GGH_3 cells.

GGH_3 cells are rat GH_3 cells, which are somatolactotropic in origin, and stably express the rat GnRHR. This cell line was engineered by cotransfecting rat GH_3 cells with the rat GnRHR expression vector (pcDNA1-GnRHR) and a pSVneo plasmid expressing the neomycin resistance gene[1]. The GGH_3 cell line is indistinguishable from its parental cell line GH_3[1]. On the GGH_3 cells there are specific, high-affinity binding sites for GnRH and GnRH analogs, although this is not present in the parental GH_3 cell line. Activation of these cells by GnRH results in the release of prolactin and growth hormone. It has been found that GnRHR in GGH_3 cells can couple to both $G\alpha_q$ and $G\alpha_s$, therefore either activating adenylyl cyclase resulting in the activation of PKA or activation of phospholipase C followed by PKC, which can result in the activation of GnRHR gene transcription [32, 33].

The advantage of this cell line is that the GnRHR's expressed in these cells couple to the same G-proteins as the endogenous GnRHR's in primary rat gonadotropes resulting in the assumption that the same signalling pathways will mediate GnRH action in both cell types [34].

1.3.2 αT3-1 cells

The αT3-1 mouse clonal cell line represents precursor pituitary gonadotrope cells. αT3-1 cells came about when a fusion gene containing a 1.8kb of the 5'- flanking sequences of the human glycoprotein hormone α-subunit was linked to the simian virus-40 (SV-40) T-antigen oncogene and was used to produce transgenic mice [35]. This resulted in tumors in the anterior pituitary and gave rise to the αT3-1 cell line. The αT3-1 cells have provided a useful cell model for the study of GnRHR and GnRH action [35]. The advantage of these cells is that they have retained several differential functions of gonadotropes, such as gonadotropin α-subunit expression, synthesis and secretion, as well as expression of the GnRHR and receptor-dependent responsiveness to GnRH [35]. These cells express the estrogen, progesterone, androgen and glucocorticoid receptors as well as the SF-1 mRNA and protein [36].

The disadvantage of these cells is that they do not express the FSH and LH β-subunit genes indicating that these cells are derived from precursor cells that were not fully differentiated into gonadotropes [35]. In αT3-1 cells the GnRHR appears to couple to the $G\alpha_s$ subunit therefore resulting in the activation of PKA and ultimately in the activation of GnRHR gene transcription [33].

1.3.3 LβT2 cells

The immortalized gonadotrope cell line (LβT2) was derived from tumors generated in transgenic mice, by a similar method to the preparation of αT3-1 cells [33, 37]. LβT2 cells are more differentiated than the αT3-1 cell line. These cells were generated later in ontogeny as compared to the αT3-1 cell line and thus represent a more mature gonadotrope precursor. In LβT2 cells the GnRHR can couple to the $G\alpha_q$ and the $G\alpha_s$ subunits. Whether the PKA and PKC pathways are involved in transcriptional regulation of the GnRHR gene in response to GnRH in these cells are not known (Sadie personal communication).

The advantage of LβT2 cells is that they exhibit more differentiated gonadotrope-like characteristics, such as the expression of both the α and β-subunit of LH and the mRNA for the GnRH receptor is expressed in this cell line. They also express the oestrogen receptors, and oestrogen inducible progesterone receptor [33]. The disadvantage of these cells is that they do not express the FSH β-subunit.

1.4 Basal regulation of GnRHR gene expression

Duval *et al.,* (1997) [14] identified a tripartite enhancer that appears to be responsible for regulating the basal levels of the mouse GnRHR gene in αT3-1 cells [14]. The tripartite enhancer included binding sites for steroidogenic factor-1 (SF-1), activator protein-1 (AP-1) and contains a GnRHR activating sequence (GRAS).

Experiments were carried out on each of the *cis*- elements mentioned above to understand what the role of each *cis*-element was in basal regulation. It was found that the *cis*-elements AP-1, SF-1 and GRAS appeared to contribute equally in the basal regulation of mGnRHR promoter [14] leading to a 60% decrease in promoter activity if any one of these sites was mutated. When combinations of any 2 cis-elements were mutated it resulted in an 80 % decrease in promoter activity. If all three sites were mutated a complete loss of basal promoter activity was seen, with the construct showing activity similar to that of a promoterless vector [14].

It has been reported that the basal regulation of the rat and sheep GnRHR genes appears to be mediated primarily through the protein kinase A second messenger pathway[14]. Researchers have also found that on the rat promoter the AP-1 site is involved in the basal promoter activity of the GnRHR gene [14].

In some pituitary cell lines such as LβT2 and GGH_3 cell lines the mouse CRE has been shown to be essential for basal promoter activity although the rat CRE does not seem to be involved in basal promoter activity in αT3-1 cells [38].

1.5 Homologous regulation of GnRHR gene transcription

Homologous regulation of GnRHR gene expression is an established mechanism for controlling the sensitivity of gonadotropes to GnRH. When a low dose of GnRH is administrated in a pulsatile fashion an increase in expression levels of the mGnRHR promoter is observed [12]. Conversely, continuous administration of GnRH at a high concentration results in a down-regulation of the GnRHR [12] via post-translational mechanisms although transcriptional mechanisms can also contribute [39, 40], as was found in αT3-1 cells. Two sites have been identified that contribute to the increase observed in GnRHR promoter transcription with continuous stimulation (4-6 hours) with 100 nM GnRH in αT3-1 cells. These sites are sequence-underlying responsiveness to GnRH-1 (SURG-1) containing binding sites for nuclear factor-Y (NF-Y) and Oct-1 [41] and sequence underlying responsiveness to GnRH-2 (SURG-2) containing the AP-1 binding site.

In αT3-1 cells the protein kinase C (PKC) pathway is involved in the GnRH response and leads to the activation of the c-Jun N-terminal kinase (JNK) pathways resulting in an increase in the expression of the AP-1 family members and the binding of these proteins to the SURG-2 site [42, 18]. SURG-1 and SURG-2 can respond independently to GnRH and the AP-1 site is important for conferring GnRH responsiveness [13], which was shown in transgenic mice [42].

In LβT2 cells an increase in the endogenous protein and mRNA levels of the mGnRHR, was revealed when these cells were exposed to long term pulsatile GnRH stimulation [43]. Other researchers found that the activity of a transfected mouse GnRHR promoter-reporter construct containing 1.2kb of GnRHR promoter in LβT2 cells showed only minimal increases when stimulated either with continuous GnRH or in a pulsatile fashion with GnRH [9].

The number of GnRHR's does appear to correlate with GnRH responsiveness [44]. In rat pituitary cultures stimulated with GnRH in a pulsatile fashion an increase in GnRHR mRNA levels was observed [45]. Suggestions have been that this mechanism involves mitogen activated protein kinase (MAPK) and maybe the cyclic 3', 5'-adenosine monophosphate (cAMP)/protein kinase A (PKA) pathways [46, 47].

Depending on the cell line different G-protein coupled receptors ($G_q/_{11\alpha}$ and G_s) can be activated through signals that occur when GnRH binds to the GnRHR. The G-proteins are described as heterotrimeric signalling molecules, which are composed of three subunits: α, β and γ. These subunits dissociate upon the exchange of GDP for GTP, which is induced by the receptor resulting in a free Gα as well as a dimer of Gβγ subunits. In the past few years many isoforms of the different subunits have been cloned and classified according to the subtype of their α subunit [48]. There are four different subtype groups, mainly G_s, G_i, G_q and G_{11}.

The coupling of different signal transduction systems to a 7 transmembrane segment receptor (TMS) is dependant on several factors, which include receptor/G-protein affinity and the density of the receptors [48]. Many factors may change the accessibility of G-proteins to receptors such as the type of cells, the concentration of the G-proteins, the density of the receptors and the compartmentalization of the signal transduction machinery [48].

Diverse downstream signalling pathways are initiated by all of the Gα subunits, as well as the dissociated βγ subunits and other receptor-interacting proteins [33, 34]. When $G_{\alpha s}$ dissociates from its βγ subunits, it exerts its action through the interaction and stimulation of adenylyl cyclase activity, which results in an increase in cAMP. The initial phase of GnRH action in certain cell lines such as GGH_3 cells and LβT2 cells involves the stimulation of phospholipase C (PLC), which is caused by the coupling of the GnRHR $G_{q|11}$ [34]. PLC leads to the production of the second messenger inositol (1, 4, 5)-triphosphate (IP3), which induces Ca^{2+} mobilization, and diacylglycerol (DAG). DAG and Ca^{2+} together can lead to the activation of PKC or MAPK [33, 34]. The binding of GnRH to the GnRHR in GGH_3 causes an influx of calcium suggesting that calcium dependant signal transduction pathways participate in the activation of the GnRHR promoter activity when GnRH is present [34].

These signals from the G-protein coupled receptors converge with the MAPK gene family, which regulates cell growth, division and differentiation through the transduction of externally derived signals [49]. MAPK translocates into the nucleus and is activated by phosphorylation. The phosphorylated MAPK in turn activates nuclear transcription factors, which are involved in DNA synthesis and cell division [36]. MAPK is involved in the regulation of the GnRHR gene and it has been shown that MAPK can be activated through GnRH in pituitary organ culture [49]. The $G_{\alpha q}$ coupled receptors stimulate MAPK in a PKC-dependent manner [50]. Reiss *et al.* (1997) [49] investigated the mechanism of activation of MAPK (ERK1 and ERK2) by GnRH in αT3-1 cells and found that the ERK1 isoform was mainly stimulated through the signalling of the GnRHR and it also involved the Ca^{2+} dependent pathway [49]. Experiments performed in cultures of primary rat pituitary cells showed that the activation of PKC increased the levels of the gonadotropin subunit messenger RNAs. Alternatively when PKC was depleted through phorbol ester treatment it resulted in a decrease in the stimulation of the LHβ-gene expression by GnRH [49].

1.6 Stress and reproduction

1.6.1 Glucocorticoids (GC)

GC are steroid hormones that are synthesized and secreted from the adrenal cortex in response to stress via the HPA axis. An example of GC is cortisol in mammals. GC are extremely important for the protection of the body against stress by regulating glucose metabolism and blood pressure [50]. An important role of GC is the dynamic modulation of the inflammatory and immune responses. The way this occurs is via cross talk with transcription factors and signalling pathways.

During the time of inflammation the lymphocytes/macrophages are activated leading to the production of inflammatory cytokines, namely tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β). In turn these cytokines activate components of the inflammatory system as well as the expression and release of corticotrophin-releasing hormone (CRH). The increase in CRH leads to the production of adrenocorticotropic releasing hormone (ACTH), stimulating the adrenal cortex, which results in the production and secretion of GC [51]. GC are anti-inflammatory hormones and have effects on many cell types including T cells, macrophages, eosinophils, neutrophils, mast cells, endothelial and epithelial cells thus creating a classical endocrine feedback loop.

The action of GC is caused by the interruption of pro-inflammatory, cytokine-mediated signalling pathways and by causing apoptosis in certain cells of the immune system. In several immune and inflammatory diseases, which include rheumatoid arthritis, inflammatory bowel disease and asthma, exogenous synthetic GC are prescribed. The biological actions of GC as anti-inflammatory agents exert their actions via the interaction of this hormone with its cognate receptor, which is a member of the nuclear receptor superfamily of proteins [51].

The regulatory role of GC occurs in many biological processes and has a great influence on many physiological functions. Using GC as anti-inflammatory and immunosuppressive drugs results in severe side effects such as osteoporosis, diabetes and psychotic manifestations.

1.6.2 The HPG-HPA link.

It appears that an activated HPA axis leads to an inhibition in the HPG axis via suppression of the GnRH pulse generator (see figure 6). HPA activation leads to the release of the neurohormone CRH. The release of CRH causes an increase in the ACTH and cortisol levels resulting in the activation of the sympathetic nervous system causing an increase in glucose levels, heart rate and blood pressure [51]. The locus cenileus/autonomic system releases norepinephrine from several central networks as a result of stress. Researchers have suggested that it could be possible that CRH is not the sole HPA neurohormone involved during the stress response as vasopressin of the paraventricular origin was found to be co-released with CRH during stress [51]. Many studies are under way to link the HPA axis to the HPG axis. Recently, it was found that when rodents and non-human primates were administered with CRH it resulted in an immediate decrease in pulsatile GnRH and LH release although this has not been the case in humans. These studies are difficult to perform in humans at it is difficult to keep the stressful conditions constant, as humans are capable of adapting to their environment [51].

In humans it is known that when the HPA axis is activated by stress or by psychological disturbances it results to an inhibition of the HPG axis (Figure 6). It has been found that if stress is severe enough that it can lead to suppression of the normal menstrual cycle, which is referred to as functional hypothalamic amenorrhea and can, lead to infertility when fully established [53]. Researchers have found it difficult to identify the threshold of stress that could interfere with the normal cycle. Many researchers have

however agreed that the final neuroendocrine event responsible in the suppression of the normal cycle is a decrease in the GnRH pulse generator resulting in a decrease in the hypothalamic GnRH activity [52] causing a decrease in GnRHR number, leading to a decrease in the LH pulse frequency and ultimately causing deficiencies in the normal menstrual cycle [52]. GC are released during stress and have been implicated in the regulation of GnRH release in the hypothalamus [54]. High levels of cortisol have also been associated in the reduction of circulating LH [54]. The mechanism behind the regulation of GnRH by GC is a suppression of GnRH in the hypothalamic area [55], which ultimately leads to a decrease in the GnRH pulse-generating centre [56]. GCs also however act directly on the pituitary, diminishing the responsiveness of the gonadotrope cells to GnRH [57].

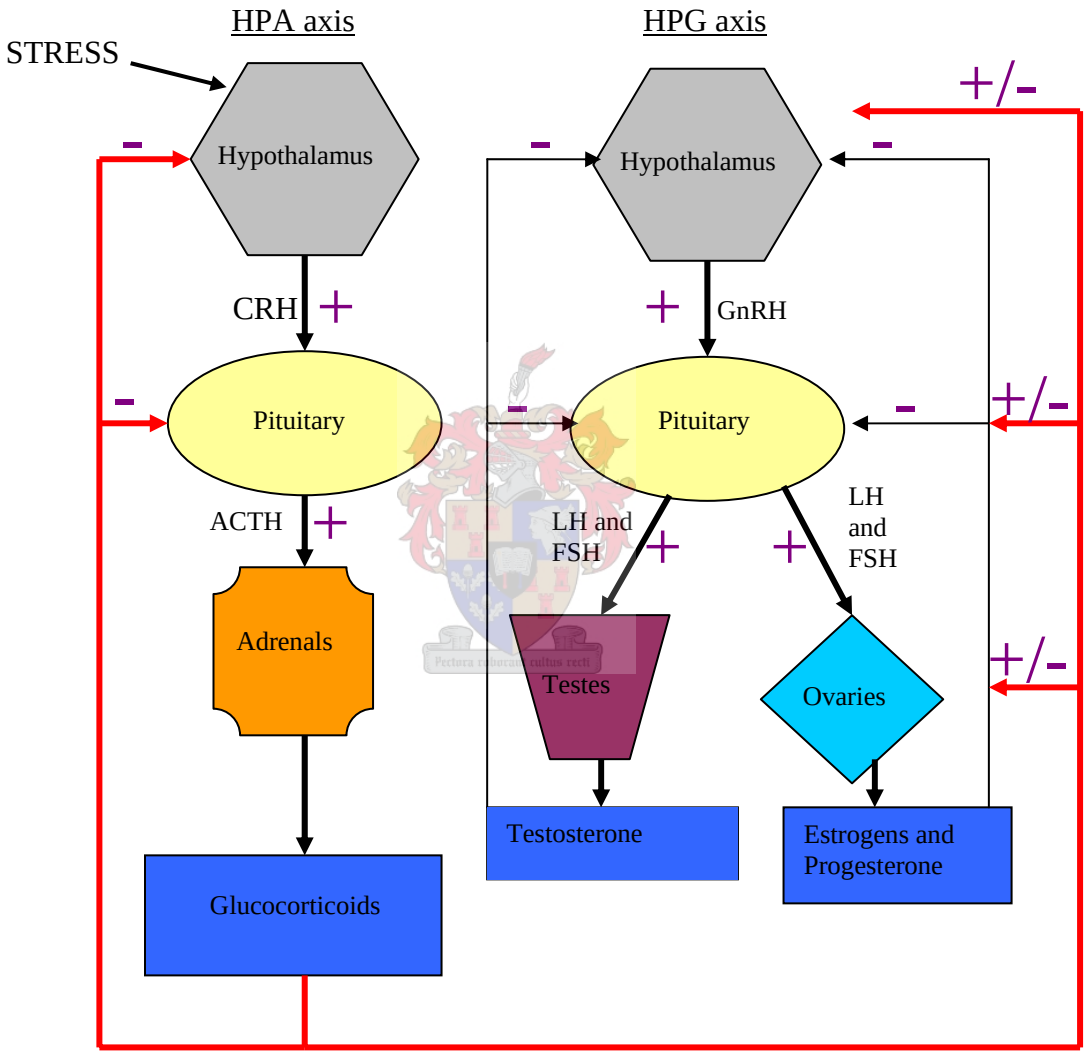


Figure 6: The HPA-HPG link and the negative/positive feedback loops between the axes. Abbreviations are as follows CRH = Corticotrophin-releasing hormone, ACTH = Adrenocorticotropic hormone, LH = Luteinizing hormone, FSH = Follicule stimulating hormone. Arrows indicate positive (+) or negative (-) feedback. Diagram by Fernandes (2006).

Researchers have said that in times of stress the HPA axis increases and the HPG axis decrease [3]. These researchers suggested that this phenomenon is attributed to preserving adrenal cortex function

at the expense of gonadal activity. At the time of stress, stress related hormones can affect the HPG axis at 3 different levels: 1) hypothalamus, resulting in the inhibition of GnRH, 2) the pituitary, which interferes with the GnRH induced LH release, 3) and at the level of the gonads altering the stimulatory effect of the gonadotropins on sex steroid secretion [3]. Therefore, stress can have both a stimulatory or inhibitory effect with regard to reproduction [58].

1.6.3 Glucocorticoid Receptor (GR)

The GR is a transcription factor which is capable of regulating several genes when its cognate hormone (GC) binds the receptor. This regulation of genes can occur either in a positive or negative way [50]. The GR belongs to the steroid hormone receptor family and is structurally similar to the progesterone (PR), mineralocorticoid (MR), androgen (AR), and oestrogen (ER) receptors [59].

The GR consists of a variable amino-terminal transactivation domain and a central and well-conserved DNA-binding domain (DBD), with a moderately conserved carboxy-terminal domain, which is responsible for ligand binding [60]. The N-terminus contains an AF-1 activation domain. This domain is important for the enhancement of transcription as well as the association of the receptor with basal transcription factors [61]. The DNA binding domain is highly conserved amongst nuclear receptors and it contains two zinc fingers, which are important for dimerization and binding of DNA. The C-terminus is where the hormone binds or when hormone is not present it is also the site where the heat shock proteins bind. There are other sites that are contained within the C-terminus such as the nuclear localization signals as well as the ligand dependent activation domain (AF-2) [61].

The human GR contains two isoforms, namely GRα and GRβ, which are generated by alternative splicing of a single gene. The difference between the two isoforms is that GRα is a 94kDa protein which consists of 777 amino acids and binds to the hormone resulting sometimes in the activation of the glucocorticoid-responsive genes whereas the GRβ is a 91kDa protein which consists of 742 amino acids and cannot bind to the hormone or any glucocorticoids [62]. Both isoforms have the first 727 amino acids in common meaning they can both transactivate and they both have the DNA-binding domains. The difference between the two isoforms is in the C-terminus. GRβ has the last 50 amino acids residues different to GRα and has a unique additional 15 non-homologous amino acid sequence [62]. This variation makes GRβ unable to bind GC resulting in no translocation into the nucleus. GRβ is however capable of binding to the glucocorticoid response element (GRE) and forms homodimers and heterodimers with GRα [62].

1.6.4 Binding of glucocorticoids to the glucocorticoid receptor

The GR is present in the cytoplasm bound to heat shock proteins (hsp90, hsp70, hsp56, hsp40) and low-molecular weight protein (p23) and many immunophilins. GCs have a lipophilic nature and therefore can easily diffuse passively through the plasma membrane and bind to the GR. Upon binding of GCs to GR, heat shock proteins are released and the GR becomes an activated transcription factor. When the

heat shock proteins are released the nuclear localization signals are exposed resulting in the rapid translocation of the ligand-bound receptor into the nucleus. In the nucleus, the ligand-bound receptor dimerizes with another ligand-bound receptor, which can then bind to the glucocorticoid-response element (GRE), which allows the receptor to interact directly or through coactivators to mediate positive or negative transcriptional responses of glucocorticoid-sensitive genes [51, 63] (see figure 7).

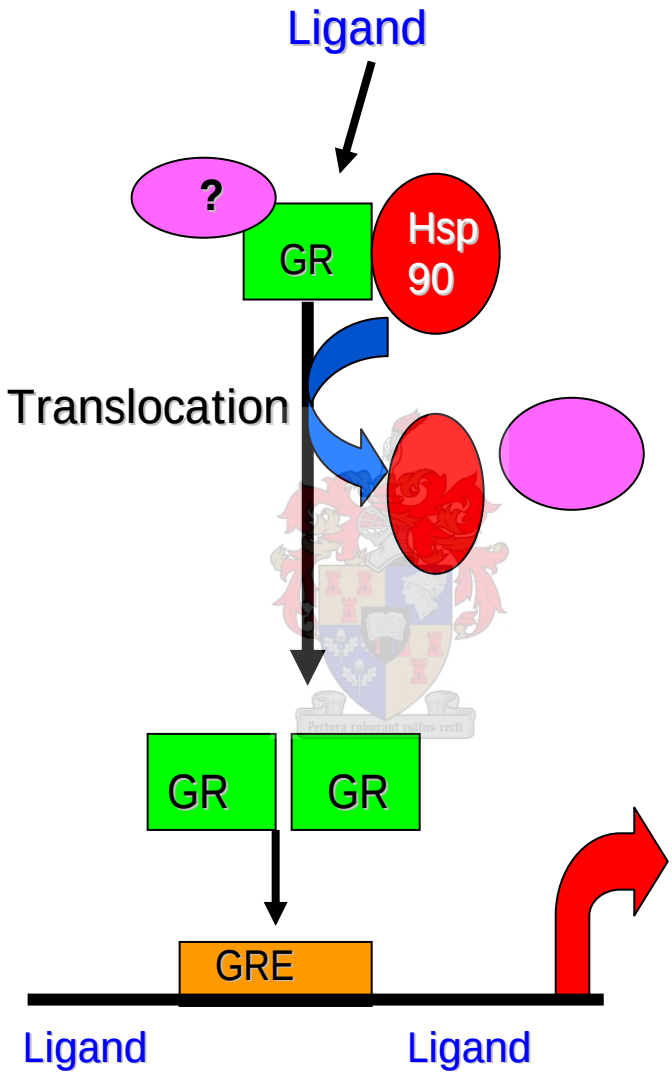


Figure 7: The mechanism of glucocorticoid binding to the glucocorticoid receptor resulting nuclear translocation leading to transactivation. Abbreviations: GR = glucocorticoid receptor; Hsp 90 = heat shock protein 90; GRE = glucocorticoid response element and ? = unknown proteins. Diagram by Fernandes (2006).

When GCs bind to the receptors they can modulate gene transcription in the nucleus in 2 ways. Firstly they can activate and secondly they can repress the transcription of target genes. Activation of transcription occurs when there is direct interaction between the GR and a GRE. However, repression of transcription can occur in two ways: 1) If there is an indirect action of GR mediated by a response

element for another transcription factor via GR-protein interaction, 2) if GR displaces another general or regulatory transcription factor [63].

1.6.5 Direct activation/repression of transcription via the GR

Once the GR is activated by GCs the activated GRs proceed rapidly to the cell nucleus and can bind as a homodimer to a GRE in the promoter region target genes (as mentioned above). This is the classical model for GR action which is characterized by the GR interacting with GREs (see figure 7). The GRE has been reported to have two defined stretches of nucleotides separated by undefined nucleotides creating two half sites. The concensus sequence of GRE is: $^{5`}$AGAACAnnnnTGTTCT$^{3`}$ where n can be any nucleotide. Therefore, the DNA binding domain of GR binds only to specifically spaced target half sites on the DNA and the specific DNA associations induce receptor dimerization. When the GR homodimer binds to the GRE the homodimer interacts with the basic transcription machinery either directly or indirectly [64].

1.6.5.1 nGRE mechanism

In some promoters an activated GR can also bind directly to a so-called negative GRE (nGRE) resulting in inhibition of transcription. It is thought that the GR binds to the nGRE and interferes with the transcriptional activation by other transactivating factors. The GR can bind to nGREs as a monomer, which leads to a suppression of gene expression as found for the keratin and CRH genes. Secondly, the GR can bind to the nGRE and antagonize the effect of a transcription factor that would naturally induce transcription. This can occur because the GR-binding site overlaps with the binding site for a required transcription factor in the promoter, which is termed competitive DNA binding [64].

1.6.5.2 Coactivators

There are also transcriptional co-activators for the GR which result in an enhanced transcriptional activity of the ligand-bound GR. Examples of these co-activators are the steroid receptor co-activator (SRC) family which consists of SRC-1, GRIP-1 and SRC-3 to name a few. Other proteins which are also involved as GR co-activators are CREB binding protein (CPB) and STAT-5. The co-activators in turn enhance transcription by recruiting secondary co-activators containing histone acetyltransferase activity (e.g. CBP) as well as recruitment of protein methyltransferases. These proteins are involved in remodelling of chromatin at the respective promoter region, thereby allowing other transcription factors to bind previously inaccessible DNA [64].

1.6.6 Indirect inhibition of transcription via the GR

Other mechanisms besides those involving nGREs can also result in GR-mediated repression, as occurs for genes which are positively regulated by pro-inflammatory transcription factors composed of dimers of Jun and Fos proteins belonging to the AP-1 family. These genes include those for cytokines and chemokines such as Interleukin-1, TNF-α, IL-4, IL-5 and IL-8. This is a so-called tethering effect which involves no direct binding of the GR to the DNA, but rather the GR binds to another transcription factor and inhibits its function, usually via blocking the recruitment of the basal transcription machinery.

Examples of these transcriptions factors are CREB, orphan nuclear receptor Nur77 65 (also known as NGF1-B) and AP-1 66 (see figure 8).

1.6.6.1 A new mechanism of transcription via the GR

An interesting scenario occurs when both GRE and an AP-1 binding site overlap to form one element with new properties which can bind both the GR as well as AP-1 proteins simultaneously. Depending on the nature of the AP-1 proteins bound, the outcome for transcriptional regulation differs. Repression is observed in the presence of the liganded-GR if only c-fos but no c-jun are present, stimulation of transcription occurs in the presence of only c-jun (c-jun homodimer), and if both c-jun and c-fos are present (c-jun/c-fos heterodimer) then repression occurs [67]. The liganded-GR remains bound to the GRE in these scenarios.

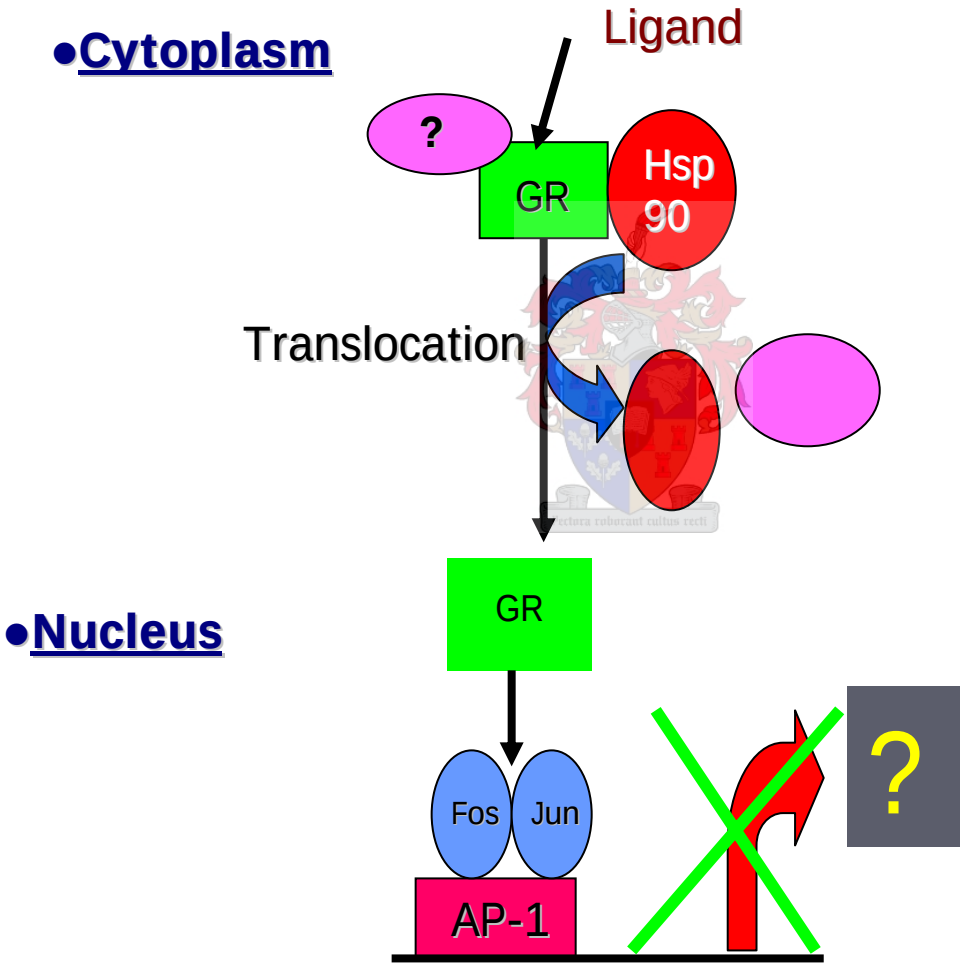


Figure 8: The tethering mechanism of glucocorticoids binding to the glucocorticoid receptor resulting in nuclear translocation leading to transrepression. Abbreviations: GR = glucocorticoid receptor; Hsp90 = heat shock protein 90 and AP-1 = activator protein-1. Diagram by Fernandes (2006).

1.6.7 Characterization of the AP-1 site and the proteins involved

AP-1 is a family of transcription factors, which can form homodimers or heterodimers of Jun (v-Jun, c-Jun, JunB, and JunD), Fos (v-Fos, c-Fos, FosB, Fra1 and Fra2) or activating transcription factor (ATF2, ATF3/LRF1 and B-ATF) and basic region leucine zipper proteins. Stable heterodimers are formed between Jun and Fos as well as between the ATF-family members. Jun and ATF proteins can also form stable homodimers with themselves. The family of AP-1 proteins typically bind to the AP-1 site (TGAGTCA). Another site that is involved is the phorbol 12-O-tetradecanoate-13-acetate (TPA)-responsive element (TRE: TGACTCA) that preferentially binds Jun-Jun and Jun-Fos dimers. The cAMP-responsive element (CRE: TGACGTCA) preferentially bind Jun-ATF and ATF-ATF proteins. Both these sites are palindromic and contain the same AP-1 half site [68]. Several new bZIP proteins have been added to the AP-1 family on the basis of the DNA sequence specificity. These are the Maf proteins (v-Maf and c-Maf) and the neural retina specific gene product (Nrl) [50]. These proteins can form heterodimers with the c-Jun and c-Fos proteins, which can then bind to the TRE, CRE, and an asymmetric DNA sequence, which consists of juxtaposed AP-1 and Maf half sites [50]. Fos forms heterodimers with the Maf related proteins. v-Jun, JunB, and JunD cannot form these heterodimers with Maf related proteins although c-Jun can form heterodimers with Maf related proteins [50]. Because of the interaction between c-Jun and the Maf related proteins novel bZIP proteins were identified, namely Jun-dimerizing partners (JDPs) 1 and 2 [50]. These proteins can bind to both the TRE and the CRE. The AP-1 site is still in its infancy stage as the numbers of bZIP proteins are increasing resulting in an increasing number of possible heterodimers that can be formed.

The abundance of the AP-1 proteins is important in the regulation of the AP-1 activity. Controlling the transcription of their genes is the most common mechanism for regulating the abundance of the AP-1 proteins. In addition, regulation can also occur at the RNA level, via protein turnover and via regulating the activity of other proteins by posttranslational modification. MAPK phosphorylates the serines and threonines of the c-Jun amino-terminal activation domain, which reduces the ubiquitination of this protein and resulting in its degradation. The opposite can occur when c-Jun is phosphorylated on serine 63 and 73 by c-Jun N-terminal kinase (JNK) resulting in an increase in stability [50]. v-Jun on the other hand is more stable than c-Jun as it is a target for JNK-mediated phosphorylation [50]. The phosphorylation of serine 63 and 73 by JNK has been found to increase the AP-1 sites transcriptional activity. It has been said that the phosphorylation step is required to allow the recruitment of the transcriptional coactivator cAMP response element-binding (CREB)-binding protein (CPB), allowing for transcriptional enhancement [50]. Enzymatic histone acetyltransferase (HAT) activity is contained within CBP and its homolog p300, which are large cointegrator proteins that have a docking platform for many different transcription factor families. HAT activity functions in such a way that it shifts the chromatin structure of the transcription factor into a looser configuration allowing access too many factors such as basal or specific transcriptional factors leading to gene transcription [50].

1.6.8 The role of AP-1

The AP-1 element is a very interesting element as it plays various roles in different cell systems. The AP-1 site has been implicated in regulating the basal levels of the GnRHR in αT3-1 cells as well as in GGH_3 cells [14, 69]. Duval *et al.* (1997) [14] found that if the AP-1 site of the mGnRHR was mutated it resulted in a 60% decrease in basal levels. Contradictory to these Cheng *et al.* (2000) [70] found that in the human GnRHR gene AP-1 does not play a role in basal promoter activity in αT3-1 cells. However, it does play a role in the decrease in human GnRHR promoter activity after GnRH treatment. They found that when αT3-1 cells were treated with GnRH it resulted in a decrease in GnRHR promoter activity, which was suggested to be most likely regulated via the PKC pathway [70]. Using various mutations in the 5‘ flanking region they found an AP-1 binding site situated at -1000/-994 within the human GnRHR 5‘ flanking region. When this AP-1 site was mutated it abolished the inhibition of the GnRHR promoter upon GnRH treatment. This implicates the AP-1 binding site in the down-regulation of GnRH [70]. Electrophoretic mobility gel shifts (EMSA’s) performed with nuclear extracts that were not treated with GnRH resulted in only the c-Jun homodimer binding to the promoter, whereas nuclear extracts that were treated with GnRH resulted in both the c-Jun and c-Fos hetero- and homodimer binding to the promoter. A suggestion was that the possible mechanism for this GnRH-mediated inhibition could be due to the composition of the AP-1 isoforms and that c-fos may play an important role in mediating GnRH induced inhibition of the human GnRHR gene expression.

Recently, experiments performed in transgenic mice expressing the GnRHR promoter that had mutations in the canonical AP-1 site (-336), showed that a loss in GnRH regulation of the mouse GnRHR promoter occurred. The results showed that an intact AP-1 site is important for GnRH responsiveness of the mouse GnRHR gene *in vivo* [42]. GnRH action on the GnRHR promoter appears to be species-specific as the sheep GnRHR promoter does not appear to contain an AP-1 consensus site [71].

1.6.9 Regulation of the GnRHR gene by steroids

Researchers have found that in rat, sheep and cow estradiol increases the level of GnRHR mRNA and proteins in the pituitary [11, 72, 73, 74, 75]. One group of researchers did however find that the GnRHR mRNA levels increased prior to the increase in estradiol leading to the conclusion that the prior decrease in progesterone was necessary in order for the up-regulation of GnRHR numbers to occur [76].

Researchers have also found contradictory results depending on the cell line. For instance McArdle *et al.* (1992) [36] found that in αT3-1 cells estradiol stimulation resulted in a decrease in GnRHR numbers, while Turgeon *et al.* (1996) [43] found that in LβT2 cells, estradiol stimulation resulted in little effect on the endogenous GnRHR gene expression levels. In many mammals that were investigated researchers found that when high levels of progesterone were present resulting in a decrease in the GnRHR protein levels leading to a decrease in the responsiveness to GnRH in the pituitary [72, 77, 78].

In αT3-1 cells it was found that when progesterone was administered in the presence of over-expressed PR isoform, an inhibition of the human GnRHR promoter activity occurred [91]. Cheng *et al.* (2001) [79] showed that this negative effect occurred via a GRE/PRE (-535/-521).

An interesting finding by Turgeon *et al.* (1996) [43] was that the endogenous GnRHR mRNA levels increased when stimulated with GCs in LβT2 cells. Further increases in the GnRHR mRNA levels were found when they pre-treated the LβT2 cells with GnRH [43]. Consistent with this result; Maya-Nunez *et al.* (2003) [69] found that GCs directly regulated transcription of the mouse GnRHR promoter in GGH_3 cells [69]. It is known that the mouse GnRHR promoter does not contain a classical GRE and that the glucocorticoid-responsiveness occurs via the AP-1 (-336) site on the mouse GnRHR promoter. Therefore, this suggests that in the presence of GCs, the liganded GR can interact with AP-1 proteins to increase GnRHR transcription [69].

1.7 Previous work completed in this laboratory

In previous research completed in this laboratory, studies were carried out on two nuclear receptor half sites termed site 1 (-15/-7) and site 2 (-244/-236) on the GnRHR promoter in αT3-1 cells [80]. When site 1 was mutated it resulted in no significant effect on the basal transcriptional activity of the GnRHR promoter. When site 2 was mutated a loss in basal activity was observed suggesting that site 2 is important in maintaining the basal levels of expression of the mGnRHR promoter.

It was shown that while the mGnRHR promoter is upregulated by PKA, neither sites 1 or 2 are required for the PKA response in these cells. However, the results did suggest a modulatory role in the PKA response for the transcription factors Steroidogenic Factor-1 (SF-1) via both sites 1 and 2.

1.8 The aim of this study

This study aimed to carry out successful attempts to mutate two *cis*-elements; namely site 3 and the AP-1 site of the mGnRHR promoter using site-directed PCR mutagenesis. Originally, this project was set out to determine whether or not SF-1 protein or AP-1 protein bound to the *cis*-elements in LβT2 cells, site 3 or the AP-1 site of the mouse GnRH receptor promoter. Anti-SF-1 and anti-fos or anti-jun antibodies would be used to confirm if SF-1 protein or AP-1 protein formed a complex with site 3 or the AP-1 site in gel mobility shift assays. Another aim of this project was to determine whether glucocorticoids and GnRH regulate transcription of the GnRHR promoter via the AP-1 site or site 3 in LβT2 cells. Other mGnRHR promoter-reporter studies were carried out by overexpressing the SF-1 protein or PKA protein and determining which *cis*-element (site 3 or the AP-1 site) is involved for the increase in mGnRHR promoter transcription.

Chapter 2: Methods and Materials

2.1 Oligonucleotides

2.1.1 Oligonucleotide sequences.

Oligonucleotides were synthesized and provided by Integrated DNA Technologies (IDT) (Whitehead Scientific, Cape Town, South Africa). The two strands of the oligonucleotides shown below in mutated site 3 were used as PCR primers in site-directed mutagenesis, as well as for electrophoretic mobility gel shifts. These sequences correspond to the mouse gene in figure 1.

Site 3:

5' *GGT CCA AG***T GTC CTT** *CCT CAC C* 3'

3' *A GGT TC***A CAG GAA** *GGA GTG G* 5'

Mutant site 3:

5' *AAT TAG AC*T CCA AG**T GT**t t**TT** CCT CAC *CTA CGA TA* 3'

3' *TTA ATC* TGA GGT TC**A CA**a a**AA** GGA GTG G*AT GCT AT* 5'

Note: The SF-1 binding site is underlined. For mutant site 3, sequences within the SF-1 site and in bold correspond to those of the wild type site 3. Letters in lower case are the sites that were mutated. The SF-1 binding site is underlined.

Nucleotides mutated were chosen on the basis of Horn *et al*. (1992) [81] where it was shown that mutation of two central C's to T's (upper strand) led to a loss of the SF-1 binding *in vitro*.

AP-1:

5' GGG ATA TTA **TGA GTC A**CT TTC GA 3'

3' C TAT AAT **ACT CAG T**GA AAG CT 5'

Mutant AP-1:

5' GGG ATA TTA g**GA** a**T**t c**CT** TTC GA 3'

3' C TAT AAT c**CT** t**A**a g**GA** AAG CT 5'

Note: Sequences in bold and underlined are the AP-1 binding site. Sequences in bold, uppercase and underlined are the wild type AP-1 consensus site. Sequences in lower case are the mutated bases of the AP-1 DNA binding site.

2.1.2 Annealing of the single stranded oligonucleotides

Equimolar ratios of the complementary single-stranded oligonucleotides were mixed with each other (10 µl of a 1 mM solution of each in H_2O) and incubated as follows: 88ºC for 2 minutes, 65ºC for 10 minutes, 37ºC for 10 minutes and 25ºC for 5 minutes. The annealing mix was then chilled on ice. The double stranded oligonucleotides were stored at -20ºC. Electrophoresis on a 2% (w/v) agarose (Whitehead Scientific) gel in 1 X TAE [94] gel was carried out in order to check the integrity and annealing of the double stranded oligonucleotides by comparing their mobility to that of the single stranded oligonucleotides.

2.2 Electroporation

Electrocompetent cells (a gift from C.Langeveldt) were diluted in a 1:1 ratio in 10% (v/v) ice-cold glycerol. 40 µl of this chilled cell suspension was added to 1 ng plasmid DNA and transferred to the prechilled electroporation (1 mm gap) cuvette and subjected to an 1800 V electric pulse in a Savant GTF 100 Gene Transformer. After the electric pulse 1 ml SOC medium was added to the cuvette to wash out the cells in the electrode gap. The cells were then transferred into a culture tube and incubated for 1 hour at 37ºC at 225 rpm on a shaking incubator. The cells were plated out at different dilutions on LB-agar plates containing 50 µg/ml ampicillin. The plates were incubated at 37ºC overnight.

2.2.1 Colony selection and glycerol stock preparation

A sterile toothpick was used to pick the positive colonies, which were suspended in 20 µl sterile water. The tube containing the cell suspension was vortexed and 400 µl LB medium containing 50 µg /ml ampicillin was added. The cells were allowed to grow overnight at 37ºC at 200 rpm on a shaking incubator. 80 µl of an 80% (v/v) glycerol solution was added to the tube containing the overgrown cells and the mixture was vortexed. The tubes containing the cells mixed with glycerol was stored at -80ºC.

2.3 Plasmid preparation

2.3.1 Wizard Plus SV Minipreps DNA Purification System

Mini-scale plasmid preparations were performed with the Promega Wizard miniprep kit. The method for the minipreps was carried out as per manufacturer's protocol, with a slight modification. Briefly, an inoculation loop was used to transfer bacterial cells from the glycerol stocks to a 10 ml LB medium containing 50 µg/ml ampicillin and cultured overnight at 37ºC with shaking at 200 rpm. The overnight culture was then centrifuged at ~11000 x g at room temperature for 5 min in a bench top microfuge. The supernatant was discarded. The cell resuspension solution 250 µl was added to the pellet which was thoroughly resuspended by pipetting. 250 µl cell lysis solution was then added to the mixture and the mixture was inverted 4 times. 10 µl alkaline protease solution was added to the inverted mixture to facilitate proteolysis and the mix was inverted a further 4 times and incubated for 5 min at room temperature. 350 µl neutralization solution was added to the mixture and mixed by inverting 4 times. The mixture was then centrifuged at ~11000 x g for 10 min at room temperature in a bench top

microfuge. The clear lysate was then decanted into a spin column and the spin column was centrifuged at top speed for 1 min at room temperature. The flow through was discarded. 750 µl wash solution (containing ethanol) was added to the spin column to wash away any contaminants and the column was centrifuged at ~11000 x g for 1 minute at room temperature. The flow-through was discarded. A second round of washing was performed by adding 250 µl of the wash solution into the spin column and centrifugation at ~11000 x g for 2 minutes at room temperature. The flow through was discarded. A modification was made to the final step. 100 µl of nuclease-free water was added to the spin column and the column was allowed to stand for 15 min before centrifugation at ~11000 x g for 1 min at room temperature. This step is performed in order to increase the yield. After centrifugation the DNA was stored at -20ºC.

2.3.2 Qiagen plasmid maxi preparation

The protocol used was in accordance with the manufacturer (Qiagen). Briefly, an innoculation loop was used to transfer cells from the glycerol stocks to a 5 ml LB medium starter culture, containing 50 µg/ml ampicillin. The starter culture was allowed to grow on a shaker for 6 hours at 200 rpm at 37ºC. 1 ml aliquots of the starter culture was inoculated into a 500 ml LB medium large culture, containing 50 µg/ml ampicillin and left to grow overnight on a shaker at 37ºC at 200 rpm. The bacterial cells were harvested by centrifugation (6000 x g for 15 minutes at 4ºC) in a Beckman JA-10 rotor. The supernatant was discarded and 10 ml of buffer P1 (containing RNase A) was added. The pellet was vigorously resuspended. 10 ml of buffer P2 was added and the mixture was gently (to ensure no shearing of the genomic DNA) inverted 4-6 times and incubated at room temperature for 5 minutes. 10 ml of pre-chilled buffer P3 was added the mixture and the mixture was mixed by gently inverting the mixture 4-6 times and allowed to incubate for 20 minutes on ice to enhance precipitation of genomic DNA, proteins, cell debris and SDS. The tube with the mixture was centrifuged at 20 000 x g for 30 min at 4ºC using a Beckman JA-20 rotor. A second round of centrifugation was carried out to make sure that all the precipitate was removed. A Qiagen-tip 500 was equilibrated by applying 10 ml buffer QBT and the column was allowed to run empty through gravity flow. The supernatant was applied to the column and it entered the resin by gravity flow. Once the column had run empty 2 x 30 ml washes with buffer QC took place to remove all contaminants. The DNA was eluted with 15 ml buffer QF. The DNA was then precipitated using 10.5 ml isopropanol at room temperature. The isopropanol was at room temperature in order to minimize salt precipitation. The precipitated DNA was then mixed and centrifuged at 15 000 x g for 30 min at 4ºC in a Beckman JA-20. The supernatant was then carefully decanted and the DNA pellet was washed with 5ml room temperature 70% (v/v) ethanol. The DNA was then centrifuged for 10 min at 15 000 x g in a Beckman JA-20. The supernatant was decanted and the pellet was air-dried for 10min. The DNA pellet was re-dissolved in 500 µl TE buffer (10 mM Tris-HCl (pH7.5), 1 mM EDTA).

2.4 Mutagenesis

2.4.1 Polymerase chain reaction (PCR) mutagenesis

pLG containing the wild type mouse gonadotropin-releasing hormone receptor promoter (~700 base pairs) was cloned into the pGL2-Basic vector (Prof J.P. Hapgood, Department of Biochemistry, University of Stellenbosch). Various bases of pLG were mutated in the present study. These mutations were of the AP-1 site (at -336/-330 relative to the translational start site) and site 3 (-304/-296 relative to the translational start site). A PCR strategy was developed to mutate the AP-1 and site 3 sites using specially designed mutant primers (refer to above section). Firstly, 400 ng of pLG was subjected to PCR using the vector primer $^{5'}$ TGT ATC TTA TGG TAC TGT AAC TG $^{3'}$ (GL1 - sense) (Whitehead Scientific, Cape Town, South Africa), which is situated in the polylinker of the pGL2-basic plasmid, along with the anti-sense mutant primer. In a separate reaction 400 ng of the pLG was also subjected to PCR but with a second vector primer $^{5'}$ CTT TAT GTT TTT GGC GTC TTC CA $^{3'}$ (GL2 - anti-sense) (Whitehead Scientific, Cape Town, South Africa), situated in the polylinker, 3' to GnRHR promoter insert, along with the sense mutant primer. Reactions were made up to a final volume of 50 µl containing 1 µl of 10 mM dNTP, 5 µl of 10x buffer, and 3.4 µl of 25 mM $MgCl_2$ (Promega) and the final volume was made up to 50 µl with water. Lastly, 1 µl Taq Polymerase (Promega) was added to the mixture. PCR was performed using a Stratagene RoboCycler. The PCR cycle was as follows: 2.5 minutes at 90°C, followed by 1 min at 93°C, 1 min at 55°C, 1 min at 72°C for 35 cycles and a last cycle of 10 min at 72°C (see figure 17).

The products from the two separate reactions were run on an analytical gel, which revealed the presence of two DNA bands both in the vicinity of ~350 bp. These fragments were gel purified by using a low melting preparative agarose gel (75 V for 30 minutes).

2.4.2 Isolation of DNA fragments using preparative low melting agarose electrophoresis

The preparative low melting agarose gels are methodologically the same as the analytical agarose gels (mentioned later) with the exceptions being the type of agarose that is used and a difference in the voltage and time of electrophoresis. Preparative gels were run at 75 V for duration of 30-45 minutes. After electrophoresis the preparative gels were analysed using a long wavelength ultraviolet light and the bands of interest were excised with a sterile scalpel.

2.4.3 Purification of the DNA fragments from the preparative low melting agarose gel

The spin column protocol of the manufacturer (Macherey-Nagel) was followed. Briefly, the fragments that were excised from the gel were placed in a sterile 1.5 ml microcentrifuge tube. The weight of the gel-excised fragment was determined. For every 100 mg of gel, 300 µl of buffer NT1 was added to the

mixture and the mixture was incubated at 50°C for 10 minutes with occasional vortexing. The sample was then loaded onto a NucleoSpin column (Macherey-Nagel) and this column was placed in a 2 ml centrifuge tube. The tubes containing the column together with the sample were centrifuged for 60 seconds at 6 000 x g at room temperature. The flow-through was discarded. 700 µl buffer NT3 was added to the spin column and the tubes were centrifuged at ~15000 x g for 60 seconds at room temperature. The flow through was discarded. A second round of 700 µl buffer NT3 was added to the spin column and the tubes were centrifuged at ~15000 x g for 60 seconds at room temperature and the flow-through was discarded. The NucleoSpin column was centrifuged again to remove all excess buffer NT3 as residual ethanol inhibits subsequent reactions. The NucleoSpin tube was placed in a fresh 1.5 ml microfuge tube and 50 µl elution buffer NE was added and the tube was centrifuged for 60 seconds at ~15000 x g at room temperature. The purified DNA fragments were contained in the flow-through and were stored at -20°C until further use.

2.4.4 PCR fusion reaction

Fusion of the two purified gel fragments (that were generated by PCR using primers GL1 sense and mutated antisense primers for one reaction, and mutated sense primers plus antisense GL2 primers in the other reaction) was carried out. The bases around the mutation site in the two fragments are complementary to each other and would thus anneal and act as primers for the fusion reaction. Equal volumes of the fragments 1 and 2 were added in the fusion reaction. The fragments were added to a final volume of 50 µl in the presence of 1 µl dNTP (10 mM), 5 µl 10 x buffer, 1 µl GL1 (20 µM), 1 µl GL2 (20 µM), 3.4 µl $MgCl_2$ (25 mM), and 1 µl *Taq* Polymerase. The samples were place into the Stratagene RoboCycler and subjected to 30 cycles. The cycles were as follows: 30 seconds at 94°C and 30 minutes at 72°C. The fusion reaction was analyzed on an agarose gel (100 V for 20 minutes) and showed a product of ~700 bp. A preparative low melting gel (75 V for 30-45 minutes) was carried out on the PCR product and the DNA fragment of ~700 bp was purified. A few additional bands were observed on the analytical gel, which could be due to non-specific binding when the PCR fusion reaction was carried out.

2.4.5 Restriction digests

An approximately 700 bp fragment was excised out of the wild type LG construct, which contained the wild type promoter. A fragment of the same size which contained all the same bases except for the mutated AP-1 site or the mutated site 3 was reinserted into the pLG construct. Restriction digests of the 700 bp PCR product was carried out with the appropriate restriction enzyme and buffers (Boehringer Mannheim, Roche) to digest DNA. The sites where the restriction enzymes cut were naturally found on the wild type construct. The restriction enzymes *Kpn1* and *Bgl II* were carried out in their appropriate buffers (buffer L and buffer H, respectively) (part of kit bought from Boehringer Mannheim, Roche) and added to both the mutated insert and the vector. The restriction digest reaction was performed in a total volume of 30 µl. The restriction enzymes were supplied in a glycerol solution; therefore the volume of enzyme added never exceeded one-tenth of the final volume. The buffers supplied were added as a 10 X dilution to the final volume. Digest samples were incubated overnight at

37°C. Double digests were performed on separate days, as the optimal salt concentrations were different. The first restriction enzyme was performed in a low salt concentration overnight and the second restriction enzyme in a high salt concentration. These conditions and the salt concentrations were also taken into consideration for the vector, LG, (2.5 µg/µl) restriction digests, which was carried out in a total volume of 20 µl. This was performed in order to excise the wild type insert, so that the mutated insert could be ligated back into the vector. After digestion, the mixture containing LG digestion products was digested with calf alkaline phosphatase to remove the 5‘ phosphate groups, in order to prevent self ligation of the vector in the subsequent ligation reaction. Briefly, the vector digest mixture was made up to a total volume of 40 µl with the following: 10 X buffer (10 X dilution), and calf alkaline phosphatase enzyme (20 x dilution). The mixture was incubated for 1.50 hours at 37°C. Digestion reactions were terminated by adding an equal volume of 2 X concentrated gel-loading buffer (3% glycerol, 0.02 M Tris (pH 6.8) and 0.1% (w/v) bromophenol blue) and analysed on an analytical agarose electrophoresis gel. The digestion products were then run on a preparative gel and the large band corresponding to the vector part of LG was excised. This was to make sure that the small fragment (the wt GnRHR promoter fragment) was not present for the subsequent ligation step (see step 2.4.7).

2.4.6 Analytical agarose gel electrophoresis

A 1% agarose gel was prepared (the percentage depends on the size of the fragments). The agarose gel was used to determine the size of the bands after restriction digestion. Preparative and analytical agarose gel electrophoresis of DNA fragments were performed according to the protocols of Sambrook *et al*. (1989) [82]. Briefly, Agarose powder (Sigma) was weighed in a 100 ml conical flask. The agarose powder was dissolved in 1 X TAE (40 mM Tris, 30 mM Acetate, 2 mM EDTA (pH 8)). The liquid agarose was heated until the agarose had dissolved. Once the mixture had cooled 10 mg/ml ethidium bromide was added to a final concentration of 0.5 µg/ml to enable the visualization of the DNA fragments under ultraviolet light. The gel solution was poured into a horizontal gel-moulding chamber (air bubbles were removed) and a suitable comb was placed in the chamber. The gel was allowed to set for 30 minutes at room temperature. When the gel was set the comb was removed and the gel chamber was placed into a horizontal electrophoresis chamber and a 1 X TAE running buffer was poured into the chamber to cover the gel. DNA containing the 1 X gel loading buffer (9 µl sample + 1 µl gel loading buffer containing glycerol) was loaded into the wells using a pipette. The gel was allowed to run for 30 minutes at 100 V (or until the loading dye had travelled to about three quarters the length of the gel). The DNA bands on the agarose gel were visualized on a UV transilluminator. Bands that appeared were bright and were caused by the ethiduim bromide in the solution, which intercalated with DNA and absorbed the UV radiation. The marker that was used was the EZ Load 100 bp PCR Molecular Ruler (AEC Amersham) resulting in bands corresponding to 100 bp, 1000 bp and 3000 bp (This marker was used when analysing PCR products). For restriction digest analysis a 0.13 µg/µl 100 bp ladder (Promega) was used with DNA fragment of sizes: 100 bp, 200 bp, 300 bp, 400 bp, 500 bp, 600 bp, 700 bp, 800 bp, 900 bp, 1000 bp and 1 500 bp.

2.4.7 Ligation

The reagents and solutions used for the ligation process were supplied with the pMOS kit (Roche). A molar ratio of 1:5 (vector: insert) was used. Insert (60 ng) (which contained the mutation of interest) was ligated into 120 ng of vector prepared in 2.4.5 in a final reaction volume of 20 µl, in the presence of 2 µl 10 X ligase buffer (1 U/mL) (Boehringer Mannheim, Roche), 2 µl T4 DNA ligase enzyme (Boehringer Mannheim, Roche). The mixture was incubated at 4°C overnight.

2.4.8 Transformation of ligated DNA into MosBlue cells

5 ng of the ligation reactions was transformed into 20 µl competent MosBlue cells (Roche Molecular Biochemicals). The mixture was incubated for 30 minutes on ice with the occasional swirl. The cells were heat shocked for 40 seconds at 42°C in a water bath; allowing DNA to enter the cell. The mixture was then placed immediately on ice for 2 minutes and 80 µl of room temperature SOC medium [82] was added. The mixture was placed on a shaker at 200 rpm for 1 hour at 37°C. The transformed cells were plated out on LB agar plates containing 50 µg/ml ampicillin and allowed to grow overnight at 37°C.

2.4.9 Screening of colonies

A PCR screening method using GL1 and GL2 primers, which are situated in the polylinker, was used. Screening of colonies is important to make sure that the colonies that have formed on the LB agar plates after transformation actually contain the insert of correct size. Colonies were picked off the LB-agar plate with a sterile toothpick and used to inoculate 20 µl sterile water and the mixture was vortexed to resuspend the colony. 5 µl of this bacterial suspension was used for PCR colony screening. The final volume for the PCR colony screening was 25 µl, which contained 10 mM dNTP, 10x buffer, 20 µM GL1, 20 µM GL2, 25 mM $MgCl_2$, and 1 µl *Taq* Polymerase (5 U/µl) (Promega) and made up to 25 µl with water. The mixture was mixed and 80 µl mineral oil was added. The PCR cycle was as follows: 35 cycles of: 1 minute at 94°C, 1 minute at 50°C, 1 minute 72°C, followed by one step of 10 minutes at 72°C after the last cycle.

The products were run on a 1% analytical agarose gel (100V; 30 minutes) and the rest of the bacterial suspension was made into glycerol stocks. Positive colonies that were identified from the agarose gel were grown up using Wizard Plus SV Minipreps DNA Purification System (Promega). 0.1 µg DNA was submitted for DNA sequencing at the DNA Sequencing facility, Department of Genetics, University of Stellenbosch, Stellenbosch using GL1 and GL2 primers.

2.5 Labelling of double-stranded oligonucleotides with Polynucleotide kinase.

The protocol for labelling was carried out as per manufacturers protocol (Roche Molecular Biochemicals). Briefly, 10 pmoles of double stranded oligonucleotide was mixed with 2 µl 10 x polynucleotide kinase (PNK) labelling buffer (part of the kit from Roche) and 20 pmoles [γ-^{32}P] ATP (AEC Amersham). One unit of PNK enzyme was added and the volume was made up to 20 µl with water. The mixture was incubated for 30 minutes at 37°C on a heating block. The DNA mixture reaction was stopped with 2.5 µl 0.2 M EDTA. The volume was made up to 100 µl with TE buffer [82]. The labelled

mixture was passed through a 1 ml G50 Sephadex (Pharmacia) spin column, which was equilibrated with TE buffer, which separated all the unincorporated nucleotides. Cherenkov counts obtained in a Beckman Scintillation Counter determined the labelling efficiency and specific activity of the probes. The specific activity was usually in the region of 10^7 - 10^9 dpm per µg labelled DNA.

2.6 Electrophoretic Gel Mobility Shift Assays.

The assay protocol for site 3 was adjusted from the method described by Horn *et al.* (1992) [81] and Sadie *et al.* (2003) [80]. Incubations for the site 3 studies were performed in final buffer concentrations of 58 mM NaCl, 0.16 mM EDTA (pH 8), 1 mM DDT, 0.4 mM PMSF, 0.25 mM EGTA, and 10 mM Tris-Cl (pH 7.5). A cocktail was prepared by adding 0.7 µl of the above mentioned buffer together with 1 µg/µl poly-dI-dC, which acts as a non-specific DNA competitor, and 20 µg/µl BSA (Roche Molecular Biochemicals) per tube reaction. 1.5 µl of the cocktail was added to 1 µl labelled oligonucleotide and 2 µg/µl nuclear extracts to form a final volume of 4 µl.

AP-1 studies were carried out according to the Strahl *et al*. (1997) [64] method. Incubations for the AP-1 studies were performed in final buffer concentrations of 1.4 mM EDTA, 0.23 mM DDT and 10 mM Tris-Cl (pH 8). 0.8 µl of the above mentioned buffer was added to 0.5 µl Poly dI-dC (Roche Molecular Biochemicals) per tube of reaction. 1 µl of this cocktail was added to 1 µl labelled oligonucleotide and 2 µg/µl nuclear extract. The final incubation volume was 20 µl. All reactions were performed with 1 µl (0.1 pmoles) radiolabelled double-stranded oligonucleotide probe (150 000 to 250 000 cpm) and 1.5 µl (1 to 2 µg) of LβT2 nuclear extracts or αT3-1 nuclear extracts. The proteins were incubated for either 10 minutes (SF-1 studies) or 20 minutes (AP-1 studies) on ice in the presence or absence of various antibodies, after which radiolabelled probe was added followed by an additional 10 minutes (SF-1) or 20 minutes (AP-1) incubation at room temperature. For antibody assays, 1 µl of undiluted antibody or antiserum was added to the mixture. In the case of rabbit pre-immune serum, 1 µl of a 1:28 dilution was added to the mixture (mixture contained no specific antibody). Pre-immune serum is added to make sure that the binding observed in the antibody reaction is specific and that other antibodies and proteins present in the rabbit pre-immune serum cannot bind to the specific site. For 30% non-denaturing PAGE, the polyacrylamide mixture (29:1 acrylamide:bisacrylamide) was prepared as follows:14,5 g ultra-pure acrylamide (USB) and 1 g N,N'-methylenediacrylamide (bisacrylamide, Merck, electrophoresis grade) was dissolved in sterile deionized water to a final volume of 100 ml. This mixture was filtered using Whatman no.2 filter paper. Thereafter the gels were poured using 6.7 ml 30% acrylamide mix, 32.5 ml MilliQ water, 400 µl 50x TAE, 70 µl TEMED and 400 µl (w/v) and 10% ammonium persulphate and allowed to set overnight at 4°C. Polyacrylamide gels were electrophoresed using a BioRad Protean II xi gel apparatus. Before loading of the samples the gels were pre-electrophoresed at 100 V for 2 hours in 1 x TAE buffer at room temperature. After the incubation of the samples, the samples were loaded onto the gel and the complexes resolved by electrophoresis at 100 V for 3.5 - 4 hours in fresh 1 x TAE buffer at room temperature. Gels were then transferred to blotting paper, wrapped in saranwrap and dried on a vacuum gel dryer, allowing the gel to dry under heat for 30 minutes followed by exposure to Hyperfilm (Amersham) at -80°C.

2.7 Tissue Culture

2.7.1 Cell culture conditions

Dr Pamela Mellon from the University of California, San Diego, California, kindly supplied LβT2 and αT3-1 pituitary gonadotrope cells. The cells were grown in Dulbecco's modified Eagle Medium (DMEM) (Sigma) (4.5 g/L glucose) with 10% fetal calf serum (Adcock Ingram) and 50 IU/ml streptomycin (GibcoBRL). The cells were maintained in 75 cm^2 cell culture flasks (Laboratory and Scientific Equipment Company (LASEC) at 37°C with 90% humidity and 5% CO_2. Cells were then plated out in 24 well plates (AEC Amersham, Nunc) at the required cell density (stated below all experiments).

2.7.2 Transfection of αT3-1 cells with the Polyethylenimine method

A 10 mM polyethylenimine (PEI) (Roche) stock was prepared in a sterile Greiner tube at pH 7. The ratio of DNA to PEI (wt/wt) was 1:10. Volumes for transfections were made up to 50 µl with 150 mM NaCl resulting in a final concentration of 150 mM. In preparation for transfections two sterile microcentrifuge tubes labelled D1 and P1 were used. D1 tubes contained 0.65 µg/ µl LG, 0.66 µg/ µl β-gal and 150 mM NaCl in a final volume of 250 µl. The P1 tubes contained 150 mM NaCl and 4.5 µl PEI in a final volume of 250 µl. Each tube was mixed vigorously and incubated for 10 minutes at room temperature. The solutions in the P1 and D1 tubes were then added together in a drop-wise fashion (PEI to DNA), vortexed and incubated again for 10 minutes at room temperature. Cells were plated out the previous day into a 12 well plate at two different densities: $1x10^5$ and $2x10^5$ cells per well. 1 ml of medium containing 10% serum was added to each well of the 12 well plates. After 10 min the medium (containing 10% serum) was removed and fresh medium containing 10% serum (1 ml/well) was added. 100 µl of the mixture (P1 and D1) was added to each well in a drop-wise fashion. Plates were incubated at 37°C overnight. The following day the medium was removed and the wells were washed with 1 ml PBS (pH 7.3) in order to wash away any excess medium and the cells were harvested. 100 µl lysis buffer (Promega) was added to each well and cells were allowed to stand for 15 minutes at room temperature. Plates were then stored at -20°C overnight.

2.7.3 Transfection of LβT2 and αT3-1 cells with the DEAE method

LβT2 and αT3-1 cells were plated out 24 hours prior to the transfection studies. The densities for experiments are stated in the legends below each experiment in the results and discussion section. The transfection medium contains 2.5% serum in DMEM, which was prepared first, since higher serum concentrations could lead to the precipitation of DEAE-dextran (1 part serum to 3 parts medium).
Chloroquine was added to the transfection medium to a final concentration of 100 µM. The transfection solution was warmed to 37 °C in a water bath. This solution was added to the different microcentrifuge tubes containing varying amounts of DNA (ranging 0.75 - 1.5 µg) diluted in distilled water. 10 mg/ml DEAE-dextran stock solution was warmed to 37 °C and mixed by inversion. 10 mg/ml DEAE (1.25 µl per single well) was added to the transfection solution. The volume of DEAE-dextran was calculated depending on the volume of DNA and the amount of DEAE to be 1.25 x number of the well, to give a final volume 500 µl, resulting in a final concentration of DEAE-dextran of 100 µg/ml. The medium in the

12-well plates were aspirated and replaced with the appropriate volume (500 µl/well for a 12 well plate) of 37 °C DEAE-dextran / DNA supplemented transfection medium. Cells were incubated for 1 hour at 37 °C. Thereafter, the transfection medium was aspirated and replaced with 1 ml of a solution containing 10% (v/v) dimethyl sulfoxide (DMSO) in phosphate buffered saline (PBS), which had been pre-warmed to 37°C. Cells were then incubated at room temperature for ~ 5 minutes. The DMSO / PBS solution was removed and the cells were washed with 1 ml PBS to remove the DMSO/PBS solution. PBS was removed and 1 ml medium (Dulbecco's modified eagle medium (DMEM) + 10% fetal calf serum (FCS) was added. The cells were incubated overnight at 37°C. The following day the medium was removed from the plates and washed with 1ml PBS (pH 7.3). The cells were harvested using 100 µl lysis buffer (Promega) and the plates containing the cells were allowed to stand for 15 minutes at room temperature. Plates were then stored at -20°C overnight.

2.7.4 Transfection of LβT2 and αT3-1 cells with lipofectamine

LβT2 and αT3-1 cells were plated out 24 hours prior to the transfection studies. The densities that were required are stated in the legends below the experiments in the results and discussion section. On the day of transfection studies the medium was removed from each well and replaced with 500 µl medium containing 10% FCS to each well of the plate. A mixture containing DNA and lipofectamine was prepared as follows: 2 microcentrifuge tubes were used, 1 tube was for DNA + medium (100 µl/well) and the other for medium + lipofectamine (Sigma) (100 µl/well). For every 1 µg of DNA, 1 µl lipofectamine was used (1:1 ratio). Depending on the volume of DNA required lipofectamine was added to medium and allowed to stand for 5 minutes at room temperature (various amounts required therefore see legends of experiments). DNA (see legends for volumes) + medium was added to the lipofectamine + medium tube and allowed to incubate for 20 minutes at room temperature. 200 µl of the mixture containing DNA and lipofectamine was added to each well of the plate containing 500 µl medium in a drop wise fashion. Plates were incubated at 37°C, overnight. The following day the cells were harvested after removing the medium and washing once with 1 ml PBS (pH 7.3) to each well, in order to wash away any excess medium. 100 µl lysis buffer (Promega) was added to each well and cells were allowed to stand for 15 minutes at room temperature. Plates were then stored at -20°C overnight.

2.7.5 Transfection of LβT2 cells with fugene

LβT2 and αT3-1 cells were plated out 24 hours prior to the transfection studies. The densities that were required are stated in the legends below the experiments in the results and discussion section. The method for fugene (Roche) was followed per manufacturer's instructions. The following day the medium was changed the and fresh pre-warmed (37°C) medium containing 10% FCS was added to each well of the plate. The required amount of DNA was added to a microcentrifuge tube (labelled 1) (see legends of experiments for volumes). The required amount of DMEM was added to a microcentrifuge tube (labelled 2). Fugene was allowed to reach room temperature. Fugene (a volume equal to twice the total amount of DNA in µg) was added to DMEM, directly to the centre of the microfuge as fugene will adhere to the sides of the microfuge and allowed to incubate for 5 minutes at room temperature.

The DMEM + fugene (tube 2) was added to DNA (tube 1) and incubated for 30 minutes at room temperature as this allowed DNA and fugene to form complexes. The growth medium of the cultured cells was replaced with fresh growth medium that contained 10% FCS. The transfection medium (50 µl/well) was added in a drop-wise fashion to the cells containing the fresh growth medium and allowed to incubate at 37°C, overnight. The following day the cells were harvested after removing the medium and washing each well once with 1 ml PBS (pH 7.3), in order to wash away any excess medium. 100 µl lysis buffer (Promega) was added to each well and cells were allowed to stand for 15 minutes at room temperature. Plates were then stored at -20°C overnight.

2.8 Luciferase studies

For all experiments e.g PEI, DEAE, lipofectamine and fugene, plates were removed from -20°C and allowed to thaw at room temperature. Once the plates were fully thawed 20 µl of the lysate of each well was removed and placed into a microtiter plate (Nunc) for luciferase assays. 100 µl luciferase assay substrate (Roche Molecular Biochemicals) was dispensed into each well. The assay substrate binds to the luciferase enzyme produced by the luciferase gene, which results in fluorescence; therefore a reading can be obtained on the labsystems luminoskan RS luminometer.

2.9 β-galactosidase studies

β-galactosidase (β-gal) assays were performed on all transfection studies e.g. PEI, DEAE, lipofectamine and fugene, as it acts as a transfection efficiency. A 50 X dilution of the Galacto-star substrate (Promega) was prepared (1 µl galactone to 49 µl diluent). Plates were removed from -20°C and allowed to thaw. Once the plates were completely thawed 20 µl of the lysate of each well was placed into a microtiter plate and used for β-gal assays. 100 µl/well of the 50 X diluted β-gal substrate was dispensed into each well and allowed to stand for 1 hour in the dark before the plates were read on the labsystems luminoskan RS luminometer. Data were normalized by dividing the luciferase values by the β-gal values.

2.10 Statistical analysis

Luciferase and β-gal assay results were graphically analysed with GraphPad PRISM™ version 4 software from GraphPad Software Incorporation. The data was analysed with one-way ANOVA, with either Dunnett's post-test (when comparing all values to a single control) or Bonferroni's post-test (when comparing all values to each other).

Results and Discussion

3.1 Nuclear proteins from LβT2 cells recognize site 3 and the AP- 1 site on the GnRHR promoter *in vitro*

3.1.1 Gel mobility shift assays

Two SF-1 regulatory cis-elements are present on the GnRHR receptor promoter. Recently a novel third putative site, site 3, with unknown function was detected by sequence analysis in our laboratory. Site 3 (-304/-296) has a similar consensus sequence as two other established SF-1 binding sites (site 1: -15/-7 and site 2: -244/-236, respectively) found on the mouse GnRH receptor promoter (see figure 1). Therefore, this specific region of the promoter was tested to see whether nuclear extracts from the LβT2 cell line contain SF-1 protein and if specific binding occurs to this sequence *in vitro*. In addition, since the AP-1 site on the mouse GnRH receptor promoter (see figure 1) was previously shown to bind c-Jun and c-Fos proteins from nuclear extracts of GGH_3 cells this lead to the investigation to whether these AP-1 proteins from the nuclear extracts of LβT2 cells could bind to the AP-1 consensus site.

Firstly, sense and antisense oligonucleotides representing the element of interest were designed, annealed, radioactively labelled and used as probes in gel mobility shift assays. The theory behind this assay is that protein-DNA complexes migrate through a non-denaturing polyacrylamide gel according to the size, charge and conformation of each individual complex. If protein binds to the specific site it significantly retards the migration of the fragment through the gel. The DNA is then “shifted” because of the protein binding. Visualization of the DNA-protein complexes can be seen on an autoradiograph due to the radiation from the DNA. DNA-specificity studies of the protein in the complex can be carried out by co-incubating excess non-labelled DNA competitors. Examples of DNA competitors can either be the same DNA fragment as the radiolabelled probe, or other sites on the promoter can be used. In order to identify the protein binding to the DNA element of interest antibodies targeted against specific proteins were used. Therefore, it is important to know what type of proteins could be involved in binding to the particular DNA element. Antibodies can cause two different types of results depending on the type of antibody that is used i.e. an ablation of shift or a supershift. An ablation of shift results when the antibody recognizes the DNA-binding domain of the protein thereby inhibiting the protein from binding to DNA leading to no complex formation. A supershift on the other hand results when the antibody can recognise the protein while in complex with the DNA.

A radioactively labelled oligonucleotide probe of site 3 was prepared and used in gel mobility shift assays, together with nuclear extracts from LβT2 cells and the SF-1 antibody. A negative control was also used which was rabbit pre- immune serum.

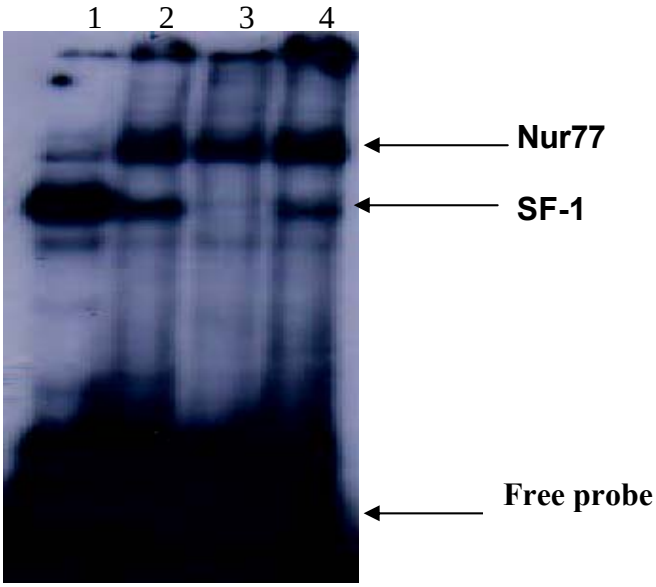


Figure 9: Autoradiograph of a gel mobility shift assay performed with the radiolabeled site 3 probe. For all lanes 1-2 µg of protein was used. Lane 1 contains nuclear extracts from αT3-1 cells. Lane 2 contains nuclear extracts from the LβT2 cells. Lane 3 contains nuclear extracts from LβT2 cells together with the SF-1 antibody. Lane 4 contains LβT2 nuclear extracts plus rabbit pre-immune serum. Arrows represent complexes containing SF-1, Nur77 protein or free probe.

The SF-1 antibody used in this experiment is directed against the DNA binding domain of SF-1 resulting in the antibody recognizing the DNA-binding domain of the protein thereby inhibiting the protein from binding to DNA, which lead to no complex formation.

Figure 9 shows that the SF-1 protein from both the LβT2 and αT3-1 gonadotrope cell lines binds to site 3. When the SF-1 antibody was present it caused an ablation of shift confirming that the protein that binds to site 3 is SF-1 (compare lanes 2 and 3). The reason why the cell line αT3-1 cells were used in this experiment is because it has previously been established that SF-1 protein is expressed in this cell line and binds to SF-1 sites 1 and 2 of the mGnRHR promoter [80]. This experiment also shows that there is more SF-1 protein in αT3-1 cells than in LβT2 cells as shown by the relative intensity of the SF-1 bands. Another band that appears on the autoradiograph is that of Nur77 as deduced by others in the laboratory.

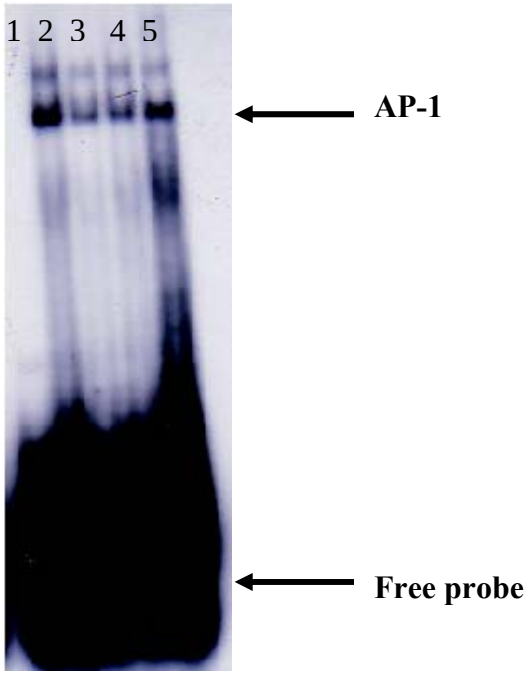


Figure 10: Autoradiograph of a gel mobility shift assay performed with the radiolabelled AP-1 probe. Lane 1 contains the probe in the absence of nuclear extracts. Lane 2 contains nuclear extracts from the LβT2 cells. Lanes 3 and 4 contain nuclear extracts from LβT2 cells together with the c-Fos antibody. Lane 5 contains LβT2 nuclear extract plus rabbit pre-immune serum. Arrows show complexes containing AP-1 protein and free probe.

This band is known to contain Nur77 as Sadie *et al.* (2003) [80] investigated this band and found that performing similar experiments as that mentioned above using Nur77 antibody resulted in the band disappearing. From the autoradiograph in figure 9 it is interesting to point out that there seems to be more Nur77 in LβT2 cells than in αT3-1 cells (compare lanes 1 and 2).

Figure 10 shows that nuclear proteins from the LβT2 cells also bind to the AP-1 site of the mouse GnRH receptor promoter (compare lanes 1 and 2). If c-Fos were present in the nuclear extracts and c-Fos antibody was added it would retard the migration and result in a supershift. When c-Fos antibody was added to the LβT2 nuclear extracts it did not cause a supershift of the DNA-protein complex. Thus it is possible that c-Fos is not present in the nuclear extracts or is present but fails to bind to the AP-1 site *in vitro* under these conditions. The former is most likely the case since the results from the literature suggests that c-Fos is not present in LβT2 cells in the absence of stimulation with GnRH [70]. Experiments were carried out by researchers [83] using micro-array analysis in LβT2 cells. Researchers found that after 1 hour of GnRH stimulation that c-Jun and JunB mRNA levels increase although JunD mRNA levels remained unchanged [83]. They also found that when they stimulated LβT2 cells with GnRH the expression levels of FosB and c-Fos increased [83]. Therefore, an experiment with the stimulation of LβT2 cells with GnRH should be carried out to investigate whether the c-Fos protein levels would increase and result in detectable binding to the AP-1 site. It is possible that other proteins such as c-Jun bind to the AP-1 site and further experiments will have to be carried out to confirm this.

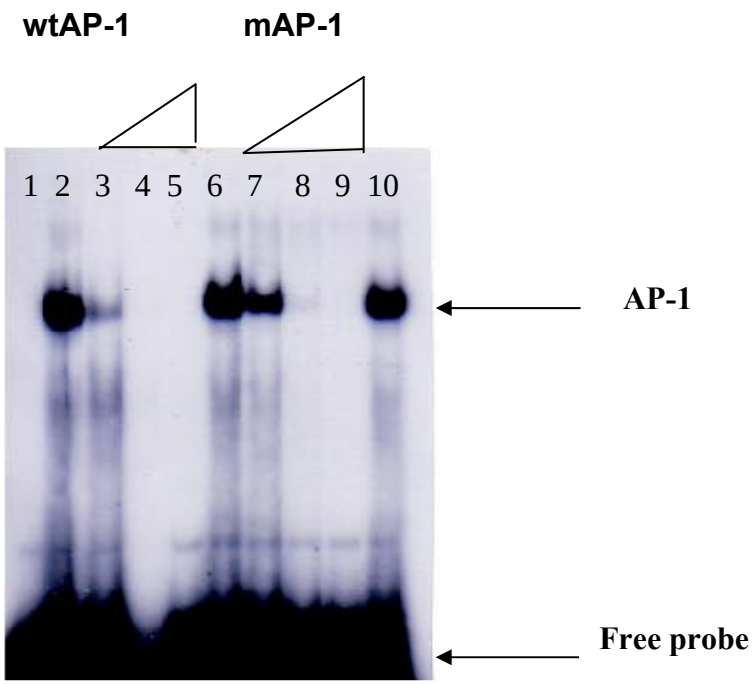


Figure 11: Autoradiograph of a gel mobility shift assay performed with LβT2 nuclear extracts and the AP-1 probe, in the absence and presence of varying concentrations of unlabelled competitor oligonucleotides. Competitors were used at 10-, 100-, and 1000-fold molar excess. Lane 1 contains the probe in the absence of nuclear extracts. Lanes 2, 6 and 10 contain nuclear extracts from the LβT2 cells in the absence of unlabelled competitive DNA. Lanes 3-5 contain nuclear extracts from LβT2 cells together with 10-, 100-, and 1000-fold molar excess, respectively of unlabelled wild type AP-1 probe (wtAP-1). Lanes 7-9 contain nuclear extracts from LβT2 cells together with 10-, 100- and 1000-fold molar excess, respectively of unlabelled mutant AP-1 probe (mAP-1). The arrows denote the AP-1 protein-DNA complexes or the free probe.

To determine the DNA-specificity of the protein(s) that bind to the AP-1 site, competitive studies of the AP-1 probe were carried out as shown in figure 11. In lanes 3-5 different molar ratios of unlabelled wild type AP-1 probe were added. Both 100- and 1000-fold molar excess of unlabelled wild type AP-1 probe resulted in the complete disappearance of the band. However, when unlabelled mutant AP-1 was added it still competed the bands away with 100- and 1000- fold molar excess. This shows that the protein(s) in the complex do not require the bases that are mutated. The AP-1 bases (wt: **T**GA **GTC** **A**CT) that were mutated for this study were **G**GA **ATT CC**T (bold letters are the bases that were mutated and the site that is underlined is the binding site). This mutation of the AP-1 site was also carried out by Maya-Nunez *et al.* (1999) [17] in studies performed in GGH_3 cells [17]. In these cells these researchers found that c-Fos and c-Jun bind to the AP-1 recognition element and when the AP-1 site was mutated a disruption of binding occurred [17]. The difference between these researchers [17] experiments and the experiments performed in this study is that Maya-Nunez *et al.* (1999) [17] stimulated

the cells with dexamethasone for 12 hours before running electrophoretic studies. In the current study nuclear extracts were prepared from cells untreated with dexamethasone. Thus it is possible that dexamethasone increases the amount of AP-1 protein allowing for easier detection compared to when unstimulated cells were used. Secondly, the binding observed in GGH_3 and not in LβT2 cells could be cell specific.

It is possible that the protein-DNA complex observed in figure 11 consists of c-Jun homodimers. A possible explanation for the binding of the c-Jun homodimers is that the homodimers do not require the bases that were mutated on the AP-1 site to be present. To my knowledge there has not been a paper published on the AP-1 sites that are important for the binding of c-Jun or c-Fos. Other proteins such as CREB and SF-1 can also interact with the AP-1 transcription proteins. Therefore, it is possible that the c-Jun protein is binding as a homodimer or with other proteins such as CREB or SF-1.

3.2 Regulation of the GnRHR promoter gene by different *cis*-elements and under different conditions

3.2.1 Optimisation of assay

The role of the -304/-296 (site 3) and -336/-330 (AP-1 site) in basal and in induced expression of the mouse GnRHR gene in LβT2 cells was investigated. Before transfection studies were carried out optimisation studies of the wild type GnRHR promoter were carried out in order to determine which transfection procedure would be optimal. The optimal conditions would be a detectable or as high as possible Relative light units (RLU's). Luciferase and β-gal values that were clearly distinguishable from background would be acceptable.

	1	2	3	4	5	6
Transfection method	lipofectamine	PEI	PEI	PEI	PEI	PEI
µg LG	1	1	0.5	0.5	1	0.5
µg β-gal	0.5	0.5	0.25	0.25	0.5	0.25

Table 1: Amounts of DNA transfected into αT3-1 cells. Experiment was carried out in duplicate.

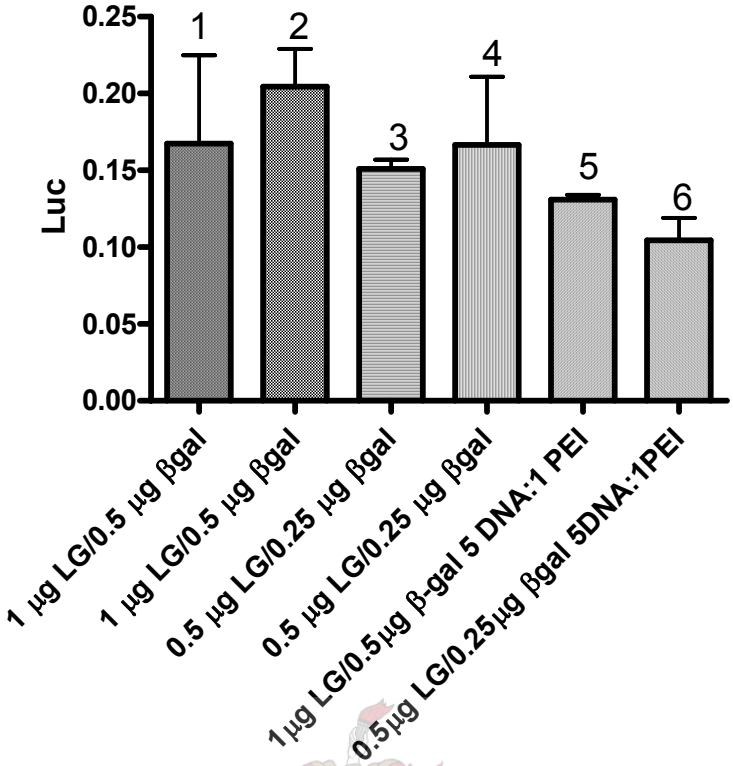


Figure 12: The effect of varying amounts of wild type LG and β-gal expression vector, ratios of DNA to PEI, and cell density on the expression of GnRHR promoter reporter using polyethylenimine (PEI) transfection method. Columns 1, 3 and 5 had a cell density of $1x10^6$ cells/well whereas columns 2, 4 and 6 had a cell density of $2x10^5$ cells/well. Values of 1 µg or 0.5 µg wild type LG were used while 0.5 µg or 0.25 µg β-gal expression vector was used. In the first column lipofectamine was used as the transfection method. In columns 2-6 the PEI method was used as the transfection method. The PEI: DNA ratio for the columns 2, 3 and 4 is 10:1. In column 5 and 6, 1 µg or 0.5 µg wild type LG was used together with 0.5 µg or 0.25 µg β-gal expression vector with a DNA: PEI ratio of 5:1. Luciferase values were shown in the graph as no β-gal values were obtained. This graph is a result of combined data from two experiments, each point in duplicate. Standard errors are shown.

Firstly, the polyethylenimine (PEI) method was investigated in order to determine if this transfection procedure would be best for transfection studies in αT3-1 cells and in LβT2 cells. Although low luciferase values were obtained, no β-gal activity above background was detected, showing very low transfection efficiency. This was found for both cell lines (αT3-1 cells and in LβT2 cells) although only the results for αT3-1 cells are shown in figure 12.

Secondly, the DEAE method was investigated.

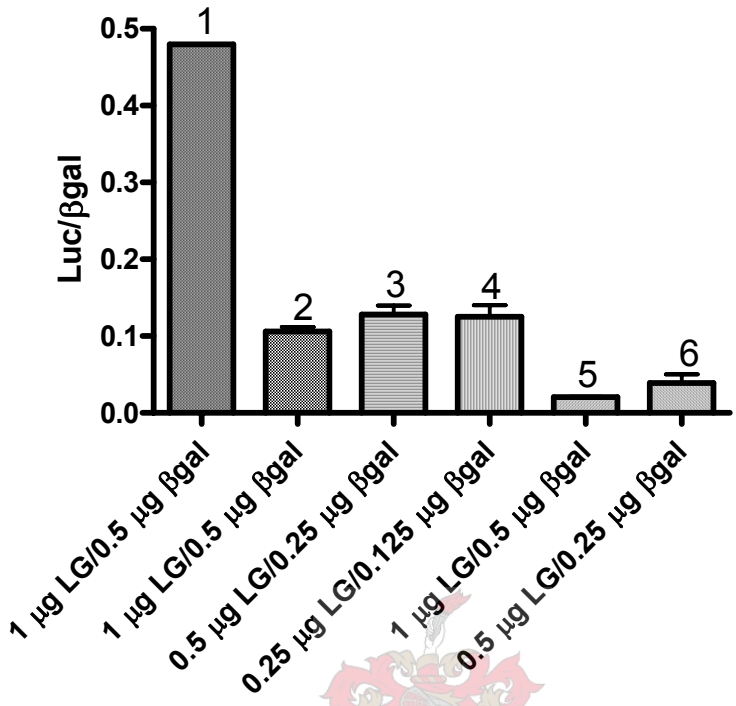
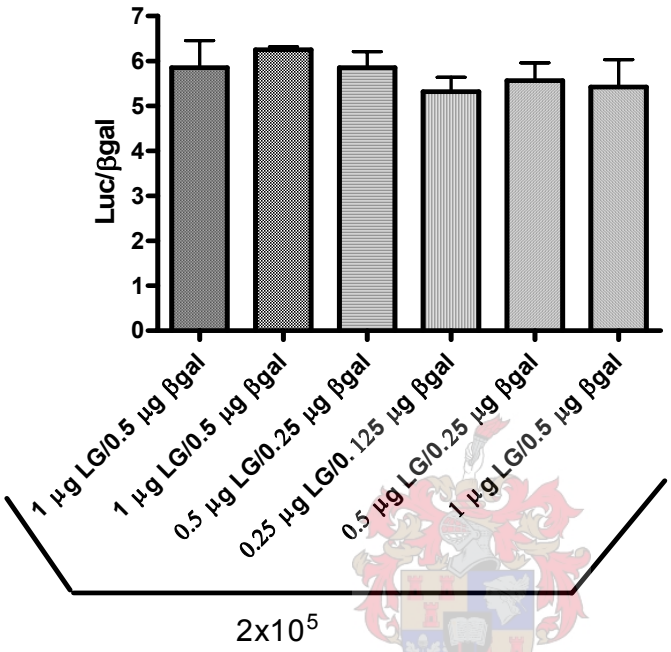


Figure 13: The effect of the DEAE-dextran transfection method with varying amounts of wild type LG and β-gal expression vector on transfection efficiency. Amounts of 1, 0.5 or 0.25 µg wild type LG were used and amounts of 0.5, 0.25, or 0.125 µg β-gal expression construct were used. In the first column lipofectamine was used to compare with DEAE-dextran. Columns 2, 3 and 4 were transfected using DEAE-dextran with a cell density of $1x10^6$ cells/well. Columns 5 and 6 were also transfected with DEAE-dextran but the cell density was $2x10^5$ cells/well. Data was normalized by dividing the Luc values by β-gal values and plotted. Standard errors are shown and the graph is a result of combined results. This experiment was carried out 3 times in duplicate.

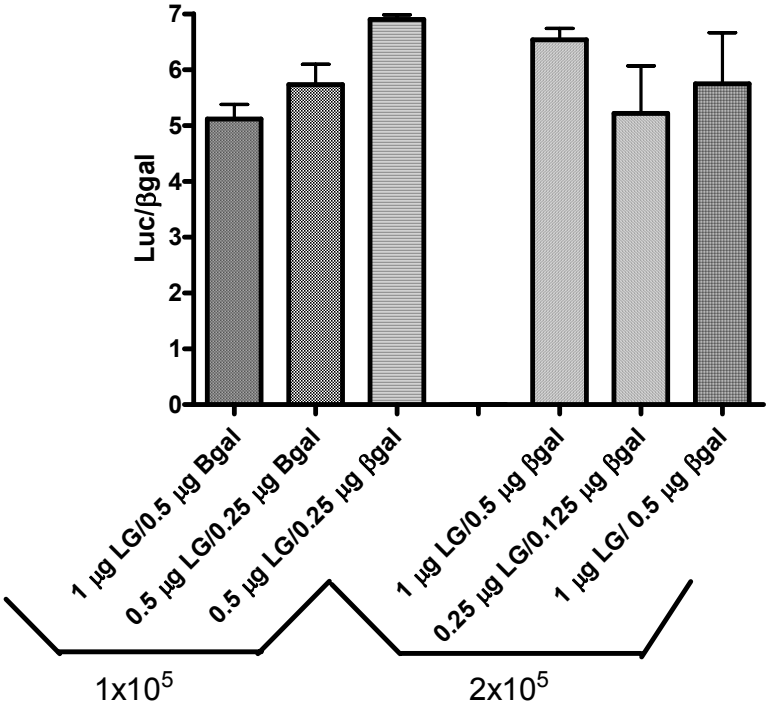
Column 1 (lipofectamine) was compared to the rest of the columns (2-6 DEAE-dextran) in figure 13. There appears to be no difference in the transfection efficiency between the different columns (columns 2-6). The transfection efficiency when one uses lipofectamine was significantly higher than when DEAE-dextran was used (compare column 1 to the rest of the columns). The absolute luciferase values that were obtained (in bars 2-6) were low (in the range of 0.46-0.67RLUs), which was similar to the PEI method. However the β-gal values were either erratic (in the range of 3.32-29.21RLUs) or could not be detected above background (in αT3-1 cells, graph not shown) showing that the DEAE-dextran method resulted in very low transfection efficiency although a slightly higher transfection efficiency than the PEI method was achieved.

In figure 13 there is a decrease in transfection efficiency when a lower cell density was used (compare columns 2 and 3 with 5 and 6). Therefore, an investigation was undertaken to determine whether varying the cell densities could result in improved transfection efficiency when using DEAE-dextran and what effect the exposure time to chloroquine would have on the transfection efficiency (Figure 14). Exposure time to chloroquine was varied, as chloroquine is toxic to the cells, which may have contributed to the low β-gal values in the previous experiment.

αT3-1 cells:- Exposure time to chloroquine for 30 minutes



αT3-1 cells:- Exposure time to chloroquine for 1 hour



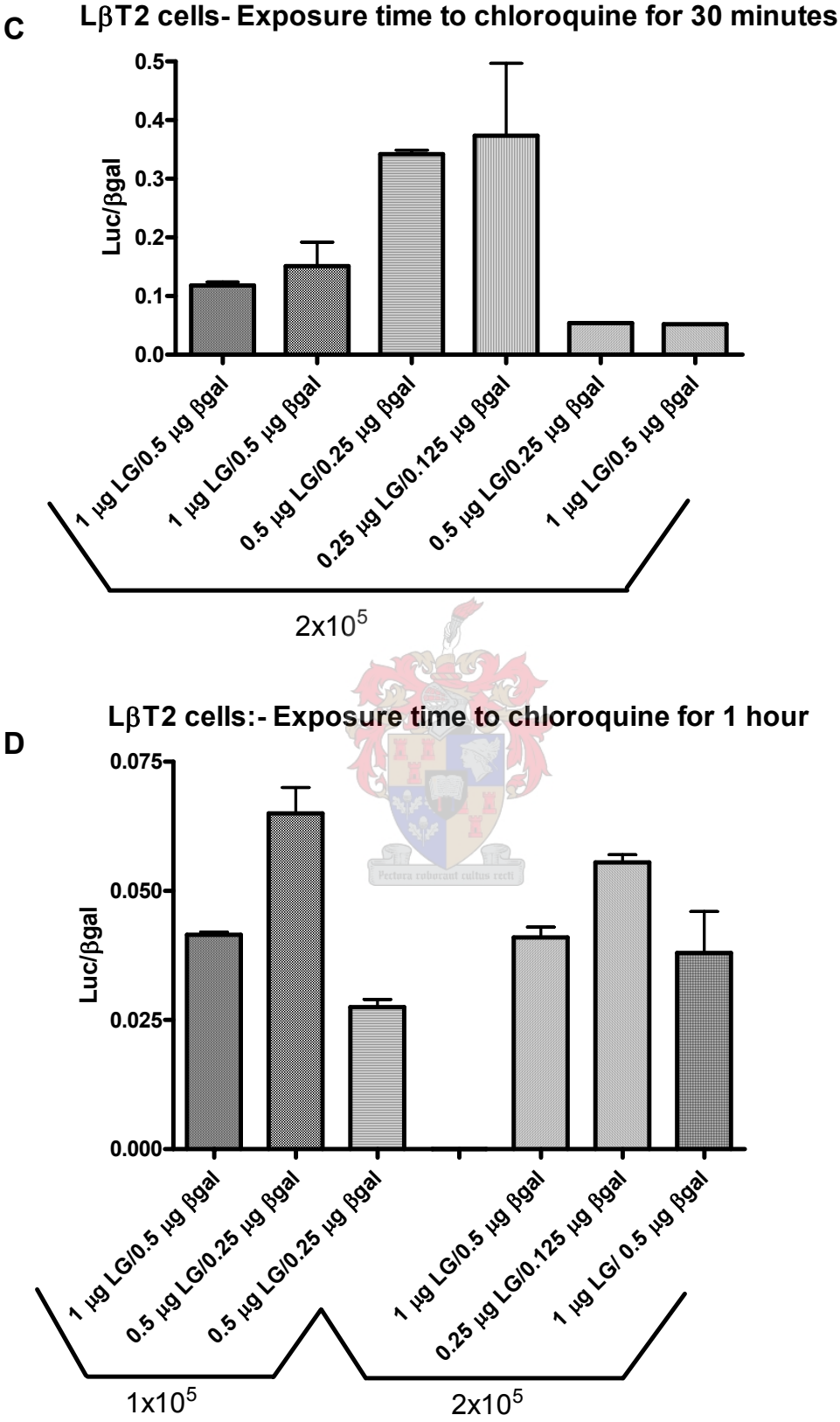


Figure 14: A comparison between LβT2 cells and αT3-1 cells using the DEAE-dextran method at varying cell densities and exposure time to chloroquine. The cell densities that were used were $1x10^5$

Figure 14 legend continuation:
cells/well or $2x10^5$ cells/well, which is indicated below the graph in brackets. The exposure time to chloroquine was 30 minutes or 1 hour, as indicated. The concentration of DEAE-dextran was also varied using 25, 12.5 or 50 µg/ml, respectively. Amounts of 1.0, 0.5 or 0.25 µg wild type LG were used together with 0.5, 0.25 or 0.125 µg β-gal expression vector. Data was normalized by dividing the luciferase values by the β-gal values. The graph is a result of combined data. Standard errors are shown and this experiment was performed twice in duplicate.

In figure 14 it appears that for the αT3-1 cells there was no difference in the luciferase expression levels i.e. transcription efficiency when one varied cell density, exposure time to chloroquine or different concentrations of DNA when using the DEAE-dextran method. In the LβT2 cells a variation of luc/β-gal values can be seen when varying amounts of DNA were added. Unfortunately, the RLU values were low and no positive control (Positive control = DNA with no promoter) was added therefore it was difficult to determine whether this was a variation due to transfection or just fluctuations in background.

The results in figure 14 show low transfection efficiency (αT3-1 cells 30 min: luc ~0.4 RLUs, β-gal ~0.07 RLUs 1 hr: luc ~0.4 RLUs, β-gal 0.08 RLUs. LβT2 cells 30 min: luc 0.4 RLUs, β-gal 0.8-9.2 RLUs 1 hr: luc ~0.6 RLUs, β-gal 7-26 RLUs), which could be due to the toxicity of chloroquine. It appears that in LβT2 cells the transfection efficiency (luc/β-gal) is lower than in αT3-1 cells, which could be due to the sensitivity of the cells to DEAE-dextran. The luciferase values that were obtained were low and the β-gal values were either erratic or low suggesting very low transfection efficiency.

Next, LβT2 cells were transfected using the fugene method in order to see if an improvement of the transfection efficiency could be detected. Firstly, the optimal ratio of DNA: β-gal was determined.

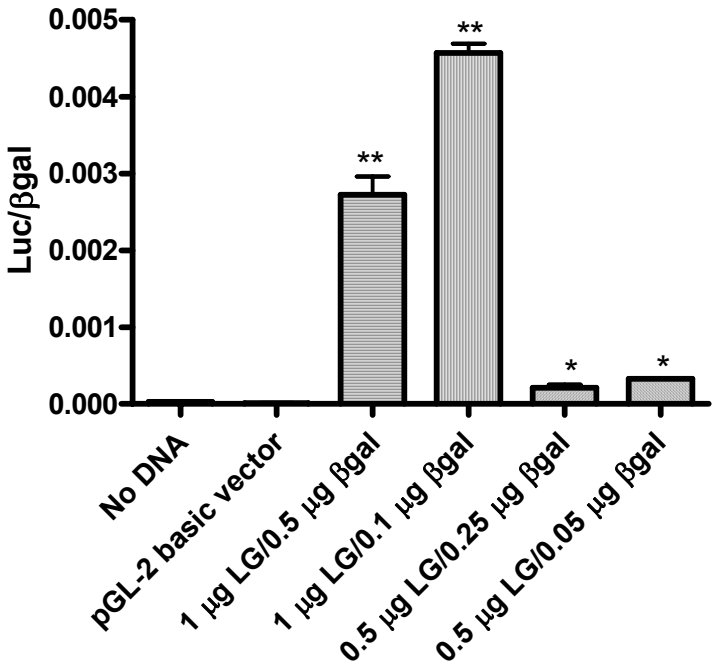


Figure 15: The fugene method with varying ratios of LG: β-gal on transfection efficiencies in LβT2 cells. Amounts of 1 µg and 0.5 µg wild type LG were transfected together with 0.5, 0.1, 0.25, or 0.05 µg β-gal. The fugene: DNA ratio was 2:1. Data was normalised by dividing the luciferase values by the β-gal values. The graph is a result of combined data. Standard errors are shown and the experiments were carried out twice in duplicate. No DNA = just cells. PGL-2 basic vector = promoterless vector. The * and ** represents the P value smaller than 0.05 (P<0.05) and 0.01 (P<0.01), respectively.

This experiment was carried out in order to determine the optimal amounts and ratios of the wild type LG and β-gal plasmids in LβT2 cells. The transfection efficiency obtained with the fugene method (see figure 15) was as follows: background for luc was 0.0098 RLUs as compared to when DNA was present of 0.19 RLUs showing a clear difference. β-gal readings showed a clear difference of ~18 RLUs when no DNA was present to ~43 RLUs (Figure 15). When a comparison of the RLUs was made between the fugene method and the PEI, DEAE-dextran or lipofectamine method it showed that fugene was the method of choice since the luciferase values were significantly higher.

From the results shown in figure 15 it was concluded that the optimal condition for future experiments was a 10 X difference between the LG: β-gal ratio. From the above experiments the optimal condition to use was 1 µg LG and 0.1 µg β-gal. Further experimentation also suggested that using 0.5 µg LG together with 0.25 µg or 0.05 µg β-gal could also be used (data not shown).

Fugene to DNA ratios were varied to determined whether a ratio of fugene: DNA could be obtained with an improved expression level of the GnRHR promoter-reporter construct. 1 µg LG and 0.5 µg β-gal was used to improve the expression level. Varying amounts of fugene was used to determine if varying the

ratio of fugene to DNA/β-gal would improve the expression levels of the GnRHR promoter-reporter construct.

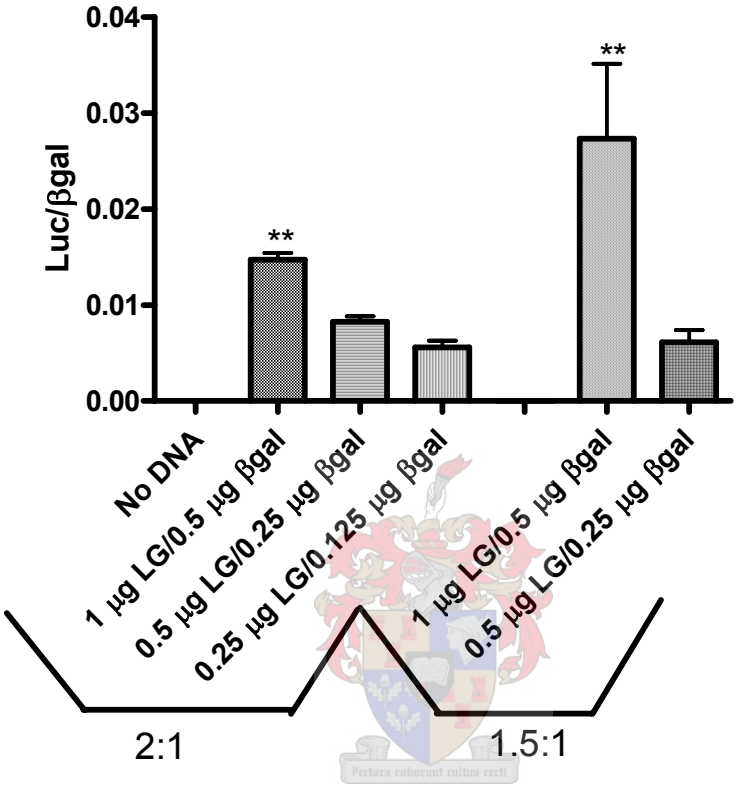


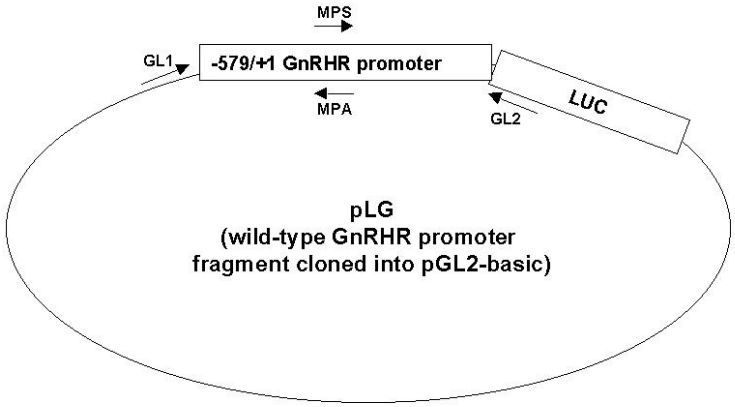
Figure 16: The effect of different fugene: DNA ratios on the transfection efficiency. LβT2 cells were used with a cell density of $2x10^5$ cells/well. Ratios of 2:1 (fugene: DNA) and 1.5:1 (fugene: DNA) were used as indicated by the brackets. Varied amounts of wild type LG promoter constructs of 1, 0.5 or 0.25 µg were used together with varied amounts of β-gal expression vectors of 0.5, 0.25 or 0.125 µg. Data were normalized by dividing the luciferase values by the β-gal values. The graph is a result of combined data. Standard errors are shown and the experiment was carried out twice in duplicate. No DNA = just contained cells. The ** represents the P value smaller than 0.01 (P<0.01).

Figure 16 shows that the highest transfection efficiency was obtained with 1 µg luc and 0.5 µg β-gal for both ratios of fugene (2:1 ratio or 1.5: 1 ratio) in LβT2 cells. However, the 2:1 ratio of fugene had a lower random experimental error/variability than to the 1.5:1 ratio. Therefore, a 2:1 ratio was used for further experiments. Thus far it is known that a 2:1 ratio of 1 µg luc and 0.5 µg β-gal resulted in the highest transcription efficiency and that one can use 1 µg LG/ 0.1 µg β-gal or 1 µg LG/ 0.5 µg β-gal.

3.3 Preparation of mutated versions of the GnRH Receptor promoter-reporter construct

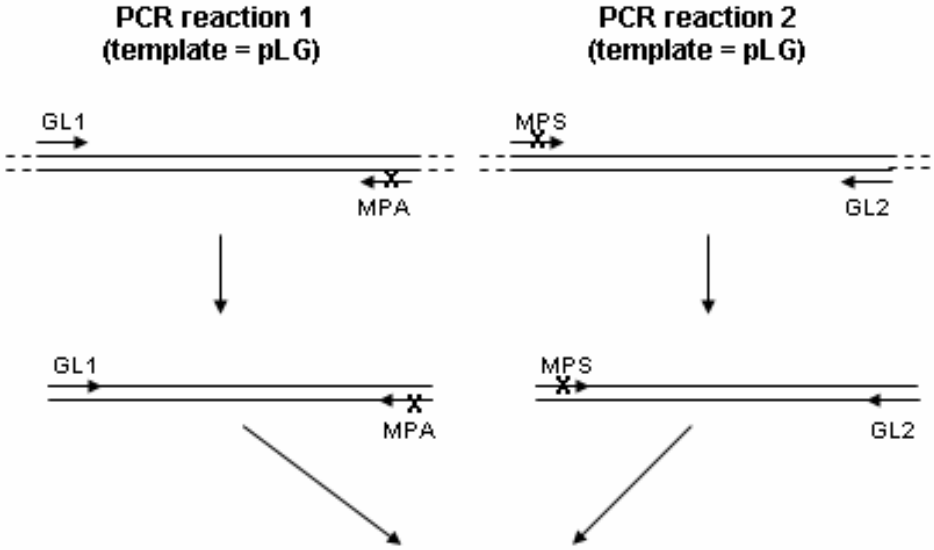
To investigate the role of the -304/-296 and -336/-330 sites in basal and in induced expression of the mouse GnRHR promoter mutant constructs were prepared by PCR mutagenesis, where the SF-1-like site 3 (-304/-296) and the AP-1-like site (-336/-330) were mutated (see figure 17). The template for site-directed PCR mutagenesis was an existing GnRHR promoter-reporter construct, which contained a GnRHR promoter fragment was cloned into the *Bgl* II site of the pGL2-basic vector (Pheiffer CP, Department of Biochemisty, University of Stellenbosch, Stellenbosch, 1998).

A

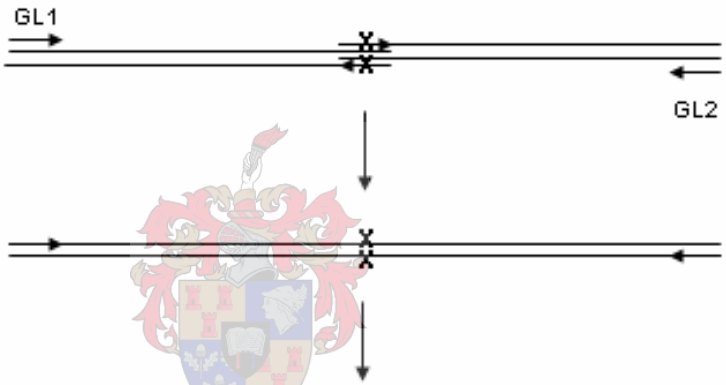


B

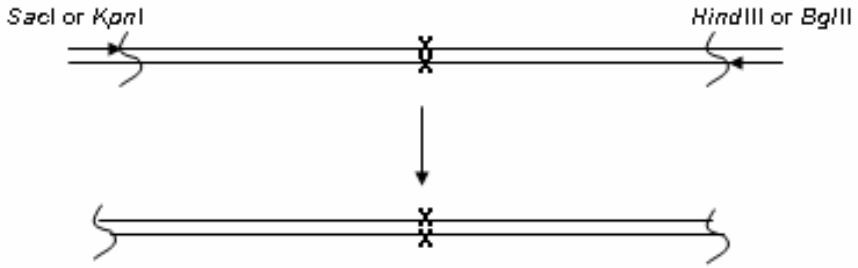
C


PCR reaction 1
(template = pLG)
PCR reaction 2
(template = pLG)
GL1
MPA
MPS
GL2
GL1
MPA
MPS
GL2

D

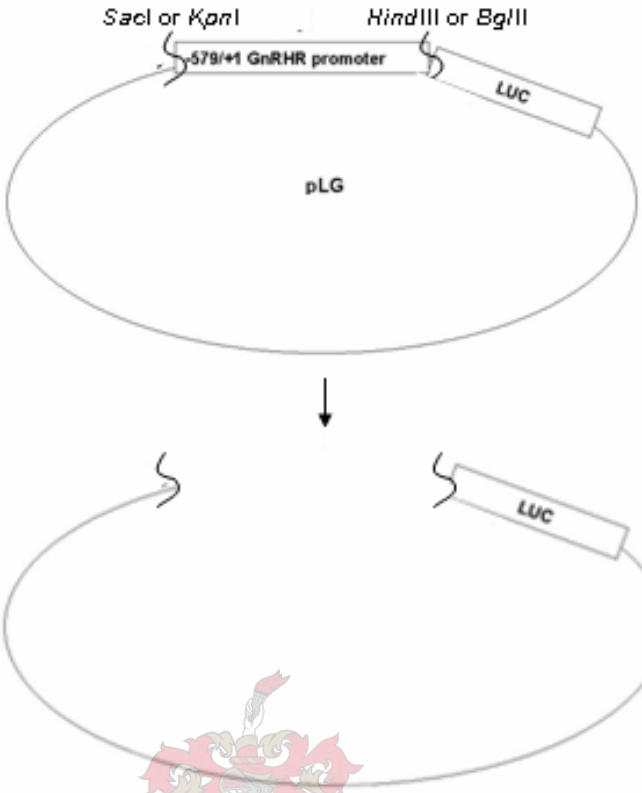

Fusion reaction
GL1
GL2

E


RE digestion, purification of insert
SacI or KpnI
HindIII or BglII

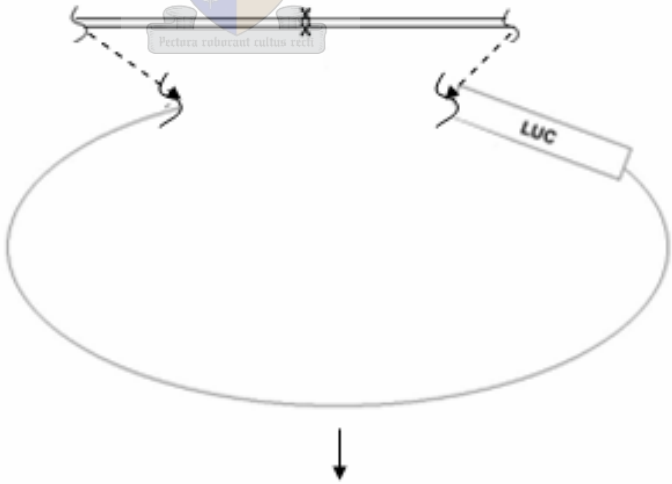
F

RE digestion, purification of vector



G

Ligation of vector and insert (3A and 3B)



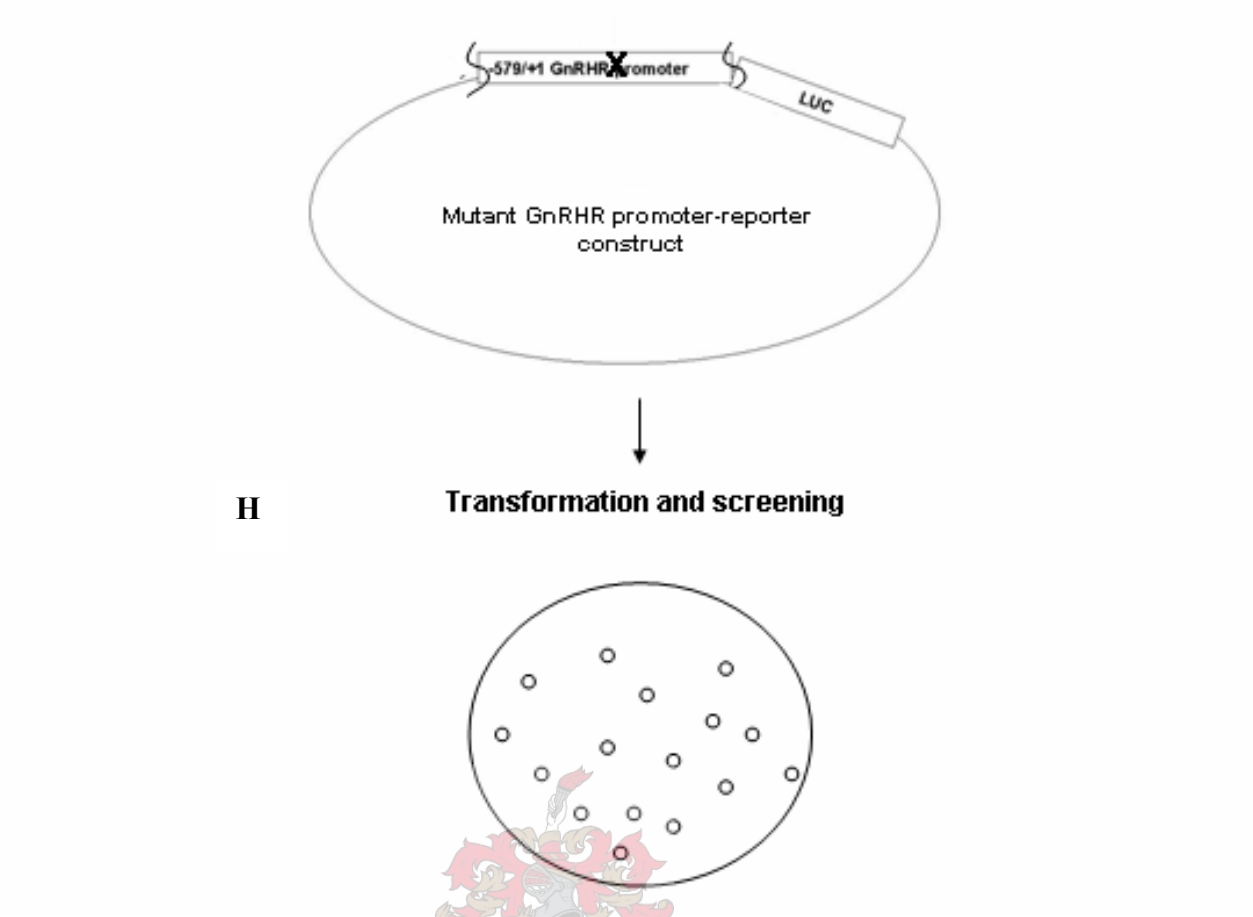
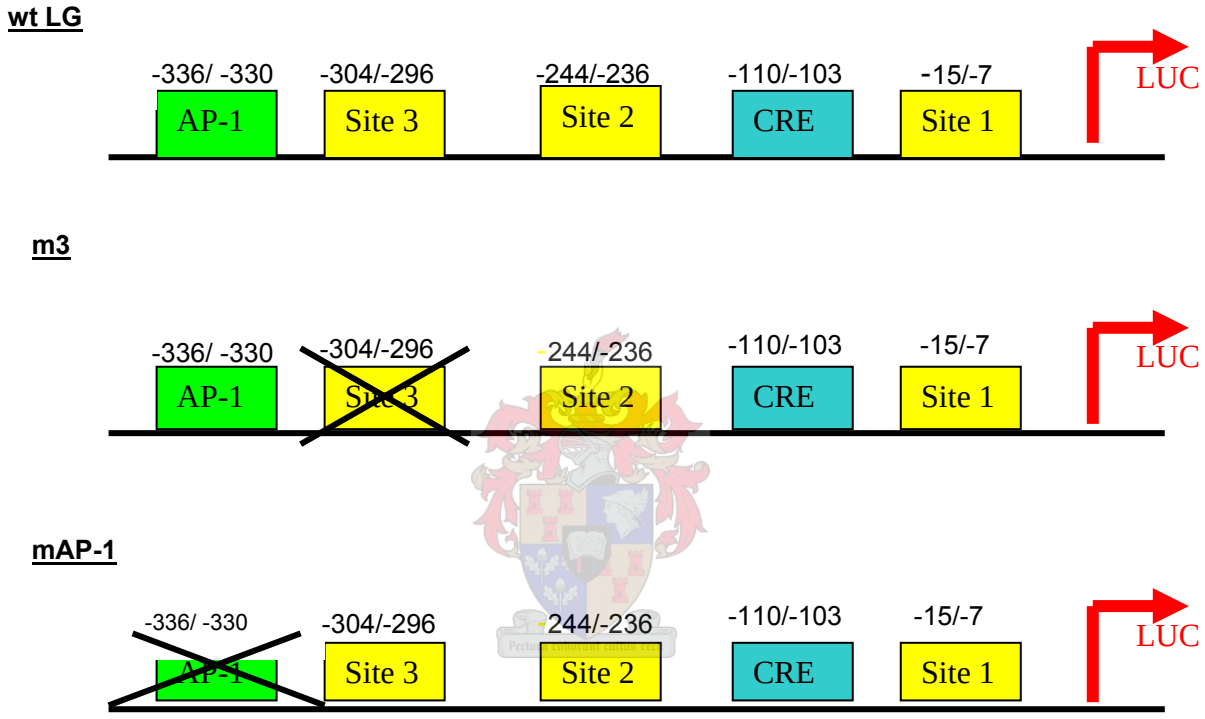


Figure 17: Schematic representation of site-directed PCR mutagenesis. A: pLG GnRHR promoter-reporter receptor construct. As shown in A is the relative GL1 (sense) and GL2 (antisense) primers, which are pGL2-basic vector-specific primers. Also indicated in A is mutated primer sense (MPA) and mutated primer antisense (MPS), which are overlapping primers containing the mutated sequence of interest. B: A detailed pLG GnRHR promoter reporter sequence together with the restriction enzyme digestion sites and the specific pGL2-basic vector-specific primers (GL1 and GL2). C: Two separate PCR reactions are carried out which includes GL1 and MPA in one reaction and GL2 and MPS in the other reaction resulting in ~300 bp and ~400 bp PCR products, respectively. D: The fusion reaction process, which is performed with the two PCR reaction products from C, which contain an overlapping mutated site. GL1 and GL2 primers are also added to the fusion reaction. The single smaller PCR product anneals at the overlapping mutated sites serving as the primer in the elongation step for the rest of the strand. E: The fusion reaction products from D are digested with restriction enzymes and gel purified. F: pLG is digested with the same restriction enzymes as used in E. Two bands are observed on the low melting gel; the smaller band (E) is the wild type insert and the larger band (F) is the pGL2-basic vector only. The digested vector was further treated with calf intestinal alkaline phosphatase to remove any 5' phosphate groups in order to prevent self ligation and gel purified. G: after gel purification of the mutated insert (as shown in E) and vector only band (shown in F) the mutated insert and vector were ligated using T4 DNA ligase. This ligated product (G) was transformed into *E.coli* MosBlue supercompetent cells and plated onto LB agar plates containing ampicillin and stored at 37°C. H: single colonies were picked and a screening process was carried out in the presence of GL1 and

Figure 17 legend continuation:

GL2 primers. After colony screening the products of the PCR reactions were analysed using a 1% agarose gel to demonstrate the presence of the correct size of insert (~700bp). For more detail see methods and materials. (Diagram taken from Sadie's PhD thesis, Department of Biochemistry, University of Stellenbosch, Stellenbosch).

The mutant constructs used in these experiments were prepared with using site-directed PCR mutagenesis (see figure 17). These constructs were designated as pLGM3 or m3 and mAP-1, which are schematically shown in figure 18



wt (site 3): TGT CCT T

m3: TGT TTT T

wt (AP-1): TGA GTC A

mAP-1: GGA ATT C

Figure 18: Schematic representation of the different GnRH receptor promoter-luciferase constructs. The wild type construct of 600 bp of the GnRHR promoter upstream of the luciferase gene was previously cloned into pGL-2 basic vector (98) known as wild type LG (wt LG). Constructs prepared from the wt LG construct but containing either a mutation in -304/-296 (i.e. m3) or a mutation in the -336/-330 (i.e. mAP-1) were prepared and are illustrated schematically. The SF-1 site 1 located at -14/-5, the SF-1 site 2 situated at -247/-236, the SF-1 site 3 situated at -304/-296 and the AP-1 site situated at position -336/-330 are shown, with the numbering all being relative to the ATG start codon. The

Figure 18 continuation:
bases that differ between wild type and mutant constructs are shown below the diagram. Luc is representative of the luciferase reporter gene.

Analytical agarose gel electrophoresis of plasmid preparations of the wild type and mutant constructs was performed (see figure 19). The plasmid preparations of wild type and mutant constructs showed that mainly supercoiled DNA was obtained. These constructs were used for transfection studies in LβT2 cells.

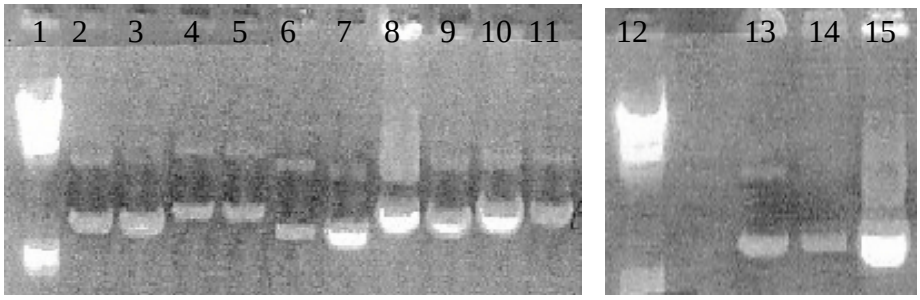


Figure 19: Analytical agarose electrophoresis of plasmids. Two different batches of the same construct were tested. Approximately 0.5-1 µg of each plasmid was loaded on a 1% agarose gel and run at 100 V for 30 minutes-1 hour. Lanes 1 and 12 contain the DNA marker (Lambda DNA/Hind III) (Promega). Lanes 2 and 3 contain the wild type pLG construct (5.6 kb vector + 660 bp insert = 6.6 kb). Lanes 4 and 5 contain the pSV-β-galactosidase (β-gal) construct (6.8 kb). Lanes 6 and 7 contain the pGL2-basic promoterless vector (Basic) (5.6 kb). Lanes 8 and 9 contain the pCMV-SF-1 construct (4.7 kb vector + 2.1 kb SF-1 cDNA). Lanes 10 and 11 contain the pFC-PKA construct (7.18 kb). Lane 13 contains the site 3 mutant of the wild type promoter (5.6 kb vector + 660 bp insert = 6.6 kb). Lanes 14 and 15 contains the activator protein-1 (AP-1) mutant of the wild type promoter (5.6 kb vector + 660 bp insert = 6.6 kb). The marker in lanes 1 and 12 was loaded incorrectly; 5 µg was loaded instead of 0.5 µg.

Lanes 2 and 3 in figure 19 contain the undigested GnRHR promoter construct, whereas lanes 13-15 contain the undigested mutated versions. These plasmids were run before on an analytical agarose gel and are presumed to be of the correct size. The positioning of the bands seems to correspond to the size of the plasmid by analysing them to each other. The lowest band on the analytical agarose gel is known as supercoiled DNA. Supercoiled pLG, m3 and mAP-1 plasmids migrate faster than the linearized plasmids (6.3 kb) because of their tightly wound structure. For transfection studies supercoiled DNA is required for optimal transfection efficiency. On the gel there are other bands present above the supercoiled bands, which are most likely, representative of linear or nicked circular plasmids.

-410 TTTCATTTTG TATCTGTCTA GTCACAACAG TTTTTAGAAA ACCTATTCAT
-360 TAAGGCTAAT TGGATGATAT TATGAGTCAC TTTCGACATC AGAATTAGAC
-310 TCCAAGTGTC CTTCCTCACC TACGATAAAA AAGACGGGGC ATCTGCTGAG
-260 GGGCTACGGT TACACTTGGC CTTCAGGAGG GCTTTGGCAT GTTCTGTTAG
-210 CACTCTTTTA GATTATAAAG GCCGAAAAAC AAGTTTACCT TGATCTTTCA
-160 CGCCAAGTCC AGAGTATCTT GGGAAAAATA AATTAGGCAG AAATGCTAAC
-110 CTGTGACGTT TCCATCTAAA GGAGGCAGAC ATCAACAGCT GCGCGTTCAG
-60 TTATGATAAA ACATCAGAAG TAACAGGGAC TCCACTCTTG
-20 AAGCCTGTCC TTGGAGAAAT **ATG**

Figure 20: The proximal mouse GnRH receptor promoter sequence. The two SF-1 sites (site 1 and site 2) are in blue. A novel third SF-1-like site (site 3) is in red. The CRE is in purple, and the AP-1 site is in orange. Numbering of the sequence is relative to the translational start site.

Once the mutations were complete the wild type pLG along with the mutants (m3 and mAP-1) were sequenced to make sure that a frame shift or deletions of certain bases had not occurred. Alignment studies of the mutant constructs were carried out on the wild type GnRHR promoter sequence (see figure 20), which confirmed no sequence differences were present except for those desired mutated bases (alignments not shown).

3.4 Site 3 and the AP-1 site are involved in the basal transcriptional regulation of the GnRHR promoter

An investigation into the role of site 3 and the AP-1 site in the basal expression of the mouse GnRHR gene was carried out. Mutated plasmids, prepared by either mutating site 3 or the AP-1 site, which are schematically shown in figure 18, were transfected into LβT2 cells and incubated overnight (16 hours).

3.4.1 The involvement of site 3 on basal transcriptional levels of the GnRHR promoter

Firstly, an investigation into the role of the SF-1-like site 3 on the GnRH receptor promoter in basal transcription in LβT2 cells was carried out. This was carried out in order to understand if site 3 played an important role in mediating the basal transcription levels of the GnRHR promoter gene. Figure 21 shows that mutating site 3 causes an increase in transcriptional activity of the GnRH receptor promoter by 1.5 fold ($P<0.01$). This increase in basal transcriptional levels indicates that site 3 plays an inhibitory role in the basal expression of the GnRH receptor promoter gene in LβT2 cells.

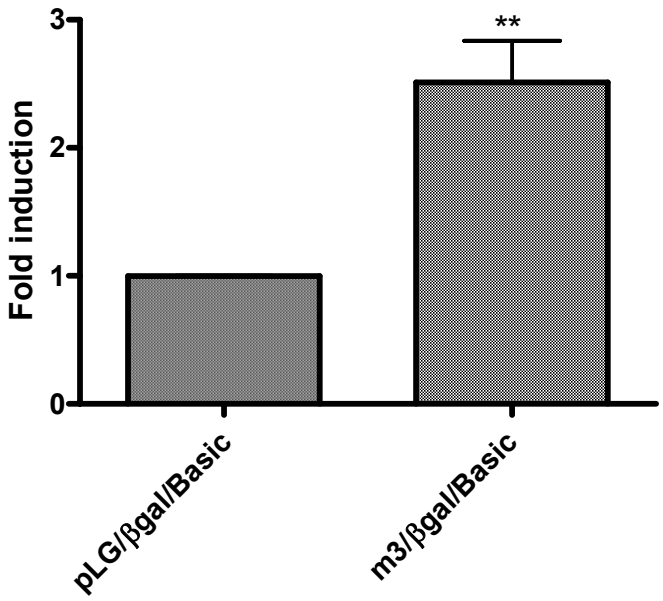


Figure 21: Role of site 3 at position -304/-296 (relative to the translational start site) on the basal transcriptional levels of the GnRHR promoter gene. LβT2 cells were transfected with wt LG or m3 both in the presence of the β-gal expression vector. Samples were transfected in triplicate. The graph is a result of combined data. Standard errors are shown and the experiment was carried out three times in triplicate. The ** represents the P value smaller than 0.01 (P<0.01) relative to wt LG. Fold induction is calculated by dividing the value obtained for m3 with that for wt LG.

Previous research in our laboratory has shown that when a similar construct containing a mutation in site 1 (pLGM1) (-15/-7 relative to the translation start site) was transfected into αT3-1 cells it showed the same basal activity as wt LG, showing that site 1 is not required for full basal activity of the GnRH receptor promoter [85]. Previous studies on site 2 (-244/-236 relative to the translation start site) showed positive regulation of basal activity of the GnRHR promoter [14, 80] in αT3-1 cells. In addition these researchers found that simultaneous mutations of both the SF-1 sites (site 1 and site 2) resulted in a 2-fold decrease in the reporter gene expression when compared to the wt LG. These results in αT3-1 cells suggests that the SF-1 sites 1 and 2 and SF-1 protein binding to these sites is important for the full basal expression of the GnRHR promoter in αT3-1 cells but that this protein can bind either to site 1 or to site 2 to maintain the basal levels and that when these sites are not available for binding that it results in a compromised basal expression [85].

The increase in basal levels obtained in LβT2 cells when site 3 was mutated suggests that site 3 is involved in repressing the basal response of the GnRHR promoter in LβT2 cells; such that when it is mutated an inhibitory element is removed. It is not known whether site 3 is involved with post-regulation of basal expression in LβT2 cells or if site 3 is involved in negative regulation in αT3-1 cells. Future studies need to be carried out in order to determine if any differences that may be observed could

possibly be due to cell-specific differences. It is evident that in both cell lines the SF-1 sites and their cognate proteins are important for basal expression levels but it is not known what the relative contribution of the different sites (1, 2 and 3) in each cell type is.

3.4.2 The involvement of the AP-1 site in basal transcriptional regulation of the GnRHR promoter

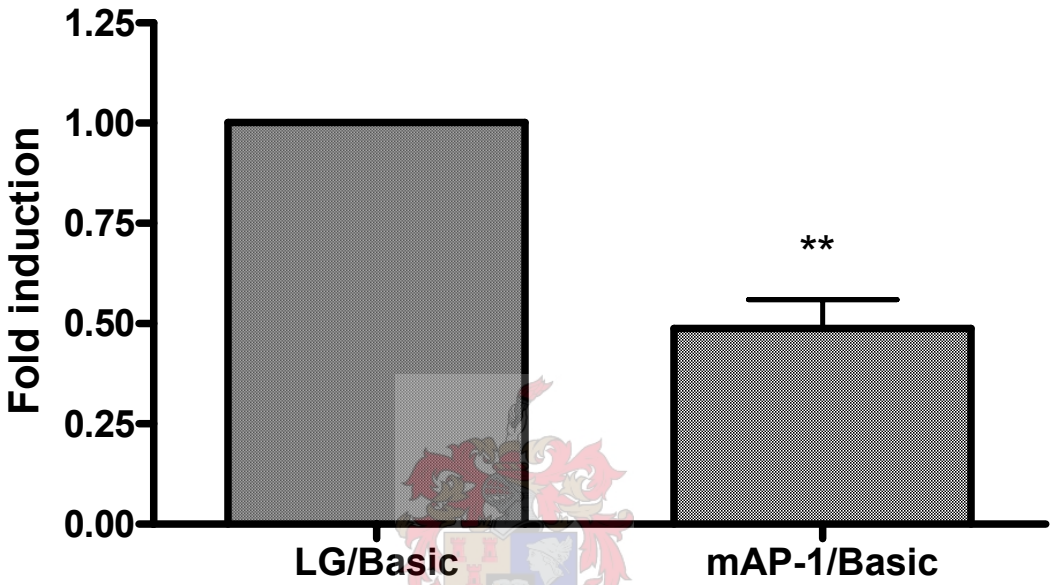


Figure 22: The effect of an AP-1 mutation on the basal transcriptional levels of the GnRHR promoter-reporter construct. LβT2 cells were transfected with either wt LG or mAP-1 both together with β-gal expression vector. The graph is a result of combined data. Standard errors are shown and this experiment was carried out five times in triplicate. The ** represents the P value smaller than 0.01 ($P<0.01$) relative to wt LG.

From figure 22 it is evident that when the AP-1 site was mutated it resulted in a decrease in the basal transcriptional levels of the mouse GnRHR promoter. This is in line with research performed by Duval *et al.* (1997) [14] and White *et al.* (1999) [18] who reported that when the AP-1 site was mutated it resulted in approximately 58% decrease in basal transcriptional levels in αT3-1 cells. Maya-Nunez *et al.* (2003) [69] also found similar results when mAP-1 of the mGnRHR promoter gene was transfected in GGH3 cells.

In the above graph there is an approximately 50% decrease in basal transcriptional levels of the mouse GnRHR promoter in LβT2 cells. Therefore, the AP-1 site is involved in positively mediating the basal transcriptional response of the GnRHR promoter in LβT2 cells.

3.5 The effect of GnRH on the GnRHR promoter

The aim was to investigate whether the proximal mouse GnRHR gene promoter responds to GnRH in a time- or dose-dependent fashion.

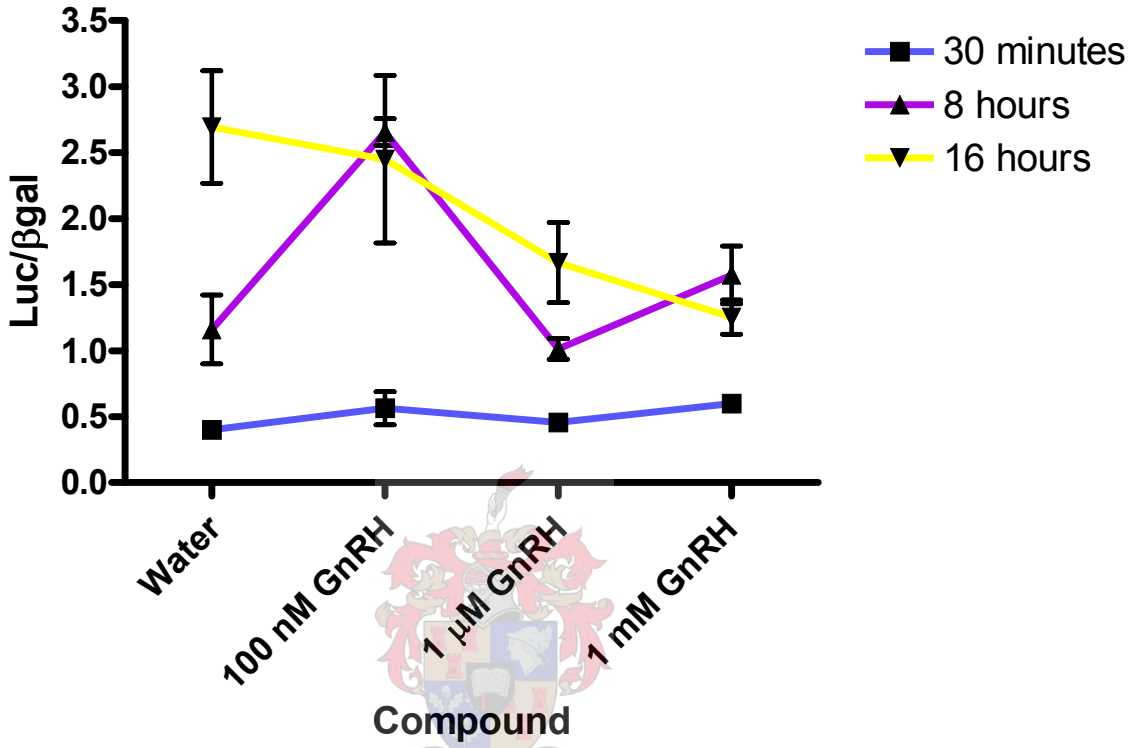


Figure 23: Time- and dose-response with GnRH on the GnRHR promoter construct in LβT2 cells. The wt LG construct was transfected into LβT2 cells using the fugene method followed by incubation for 24 hours. The following day the medium was removed and the cells were stimulated with 100 nM, 1 µM or 1 mM GnRH for 30 minutes, 8 hours and 16 hours. After the required times the cells were harvested and assayed. Data was normalized by dividing the luciferase values by the β-gal values. The graph is a result of combined data. Standard errors are shown and the experiment was carried out in five independent experiments in triplicate. It is shown from the graph that the best dose- and time point is 100 nM for 8 hours.

In figure 23 when LβT2 cells were stimulated with GnRH with varying times and doses an increased expression at 100nM at 8 hours and 16 hours with the greatest increase at 100 nM at 8 hours was observed. Cells that were stimulated for 30 minutes had no change in transcription of the GnRHR promoter. When cells were stimulated for 16 hours with 100 nM, 1 µM and 1 mM, a decrease in transcription was observed suggesting that the cells could be reverting to a negative feedback loop resulting in a decrease in the response of the cells to GnRH. What this means is that because GnRH is present for a lengthy period the transcription machinery starts switching off as it is over stimulated. It could also mean that for this period of 16 hours GnRH could be toxic resulting in cells dying.

3.5.1 Is site 3 involved in the GnRH regulation of the GnRHR promoter?

Site 3 and the AP-1 site were investigated to see if they contributed in any way to the stimulatory effect of GnRH on transcription of the GnRH receptor gene in LβT2 cells. Results shown in figure 24, show the effect of stimulation of the wild type GnRHR promoter with 100 nM GnRH for 8 hours, which resulted in a 2.5 fold increase in transcriptional activity. Mutated site 3 was also transfected into LβT2 cells without GnRH stimulation and resulted in a 1.5 fold increase in basal transcriptional levels as shown before in figure 21.

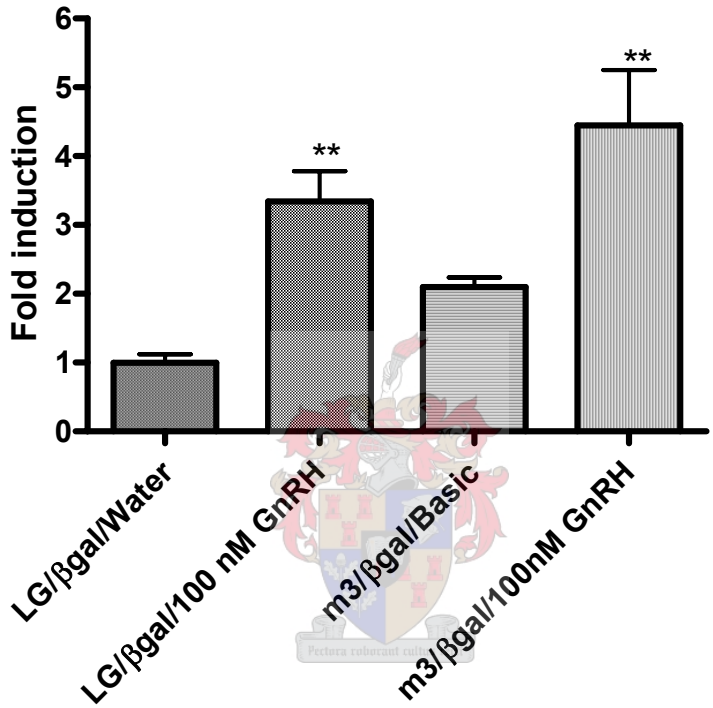


Figure 24: A representative graph of the role of site 3 in transcription of the GnRHR promoter in LβT2 cells. The cells were stimulated either with or without 100 nM GnRH for 8 hours together with wt LG or mutated site 3 and the β-gal expression vector, before harvesting. The graph is a result of one representative out of three experiments. Standard errors are shown and this experiment was carried out three times in triplicate. The ** represents a P value of less than 0.01 ($P<0.01$), relative to the control (i.e. first bar).

When cells containing the mutated site 3 construct were stimulated with 100 nM GnRH for 8 hours an increase in transcriptional levels of approximately 2.5 fold above basal levels (compare bars 1 and 2 to 3 and 4) was observed. Therefore, the mutated site 3 construct retained a similar degree of responsiveness to GnRH as wt LG. Therefore, site 3 does not seem to be involved in the GnRH responsiveness of the GnRHR promoter. Others have shown that the SF-1 site 2 is not involved in the GnRH response in αT3-1 cells [80]. This is in line with the findings for site 3, which is not involved in the GnRH response in LβT2 cells.

3.5.2 Is the AP-1 site involved in the GnRH regulation of the GnRHR promoter?

The AP-1 site was investigated to see if it played a role in the GnRH responsiveness of the GnRHR promoter in LβT2 cells (Figure 25). Wt LG and a construct containing the mutated AP-1 site were stimulated with 100 nM GnRH and allowed to incubate for 8 hours before harvesting the cells with a lysis buffer.

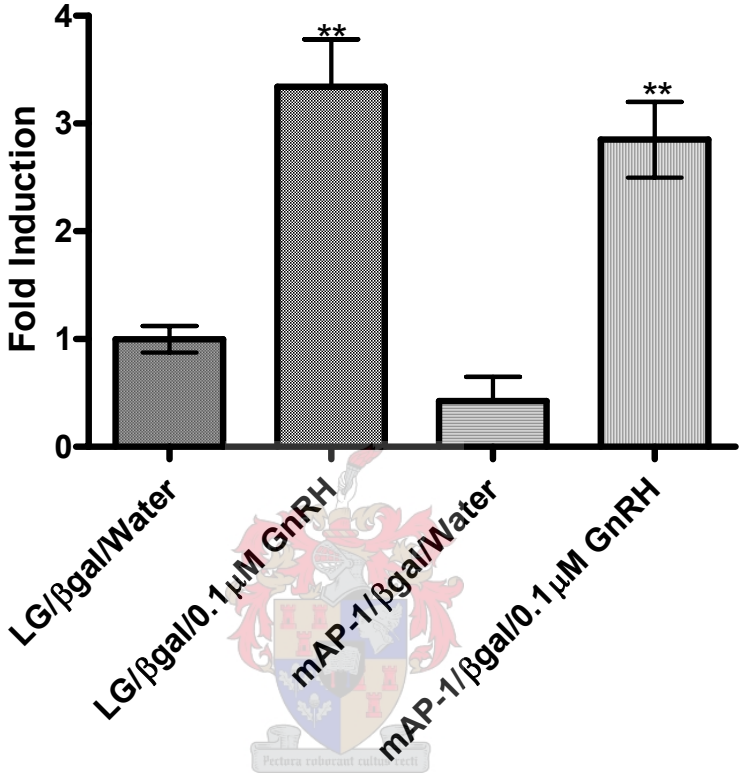


Figure 25: A representative graph of the role of the AP-1 site in transcription of the GnRHR promoter in LβT2 cells. The cells were stimulated either with or without 100 nM GnRH for 8 hours together with wt LG or mutated AP-1 and the β-gal expression vector, before harvesting. The graph is a result of one representative out of five experiments. Standard errors are shown and this experiment was performed 5 times in triplicate. The ** represents a P value of less than 0.01 ($P<0.01$), relative to the control.

When cells containing the wt LG promoter were stimulated with 100 nM GnRH an increased transcription of the GnRHR promoter of approximately 2.5 fold above basal levels (compare bars 1 and 2) was observed. When the construct containing the mutated AP-1 site was transfected into LβT2 cells basal transcriptional levels decreased by 50%, although when stimulated with 100 nM GnRH this treatment increased transcriptional levels by approximately 2 fold (figure 25, compare bars 3 and 4). It appears that the fold increase in the expression levels caused by GnRH was similar for both wt LG and mAP-1 leading to the deduction that the AP-1 site is either not involved or has a minor effect on the GnRH-responsiveness of the GnRHR promoter.

Studies carried out on GnRH regulation are not always consistent between different cell lines or sometimes even in the same cell line. For instance some researchers observe an increase in GnRHR numbers in rat pituitary cells when stimulated with continuous administration of GnRH over several hours [86], whereas others observed no effect with continuous administration of GnRH but found an increase in GnRHR mRNA levels with pulsatile GnRH stimulation[1]. What this means is that the method of administration affects the response.

White *et al.* (1999) [18] found that when using a similar construct containing a mutated canonical AP-1 element on the murine GnRHR promoter, a loss in GnRH responsiveness is obtained in αT3-1 cells. Ellsworth *et al.* (2003) [42] found that the induction of the murine GnRHR gene expression in αT3-1 cells was partially mediated by the same AP-1 site via the PKC pathway [42]. These researchers also found that when the AP-1 site was selectively removed in transgenic mice that it led to a loss of GnRH regulation of the murine GnRHR promoter. The problem with comparing these results found by Ellsworth *et al.* (2003) [42] in αT3-1 cells with those obtained in the present study in LβT2 cells is that the GnRHR appears to couple to different G-proteins in the two cell-lines, and hence activate different kinases, which may explain the different results.

3.6 The effect of PKA on the GnRHR promoter; is site 3 and or the AP-1 site involved?

An investigation into whether the activation of the PKA pathway regulated the GnRHR promoter gene in LβT2 cells was carried out together with the role of site 3 and or the AP-1 site in this activation. The PKA protein was over-expressed in LβT2 cells using transient transfections of an expression vector for PKA. Secondly, functional studies were carried out to provide functional evidence for the role of the SF-1 protein as previous experiments suggested that both site 3 and the AP-1 site are involved in regulation of basal expression levels of the GnRHR promoter gene in LβT2 cells (See figure 21 and 22 respectively). The strategy used was over-expressing the SF-1 protein using transient transfections of an expression vector for SF-1 protein to determine its involvement via site 3 or the AP-1 site.

Site 3 and the AP-1 site were investigated to determine if these *cis*-elements contributed in any way to the stimulatory effect of the PKA pathway via the 600 bp of GnRH promoter region in LβT2 cells. GnRHR promoter constructs or mutated version thereof were transiently transfected in the presence of the PKA expression vector into LβT2 cells.

3.6.1 Is site 3 involved in the PKA response?

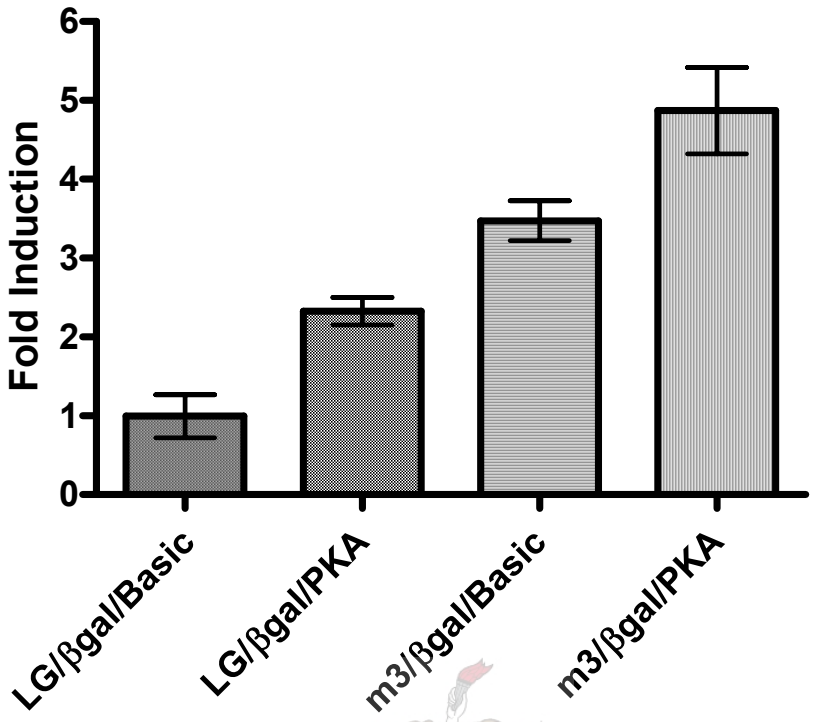


Figure 26: The effect of overexpression of PKA on the GnRHR promoter in LβT2 cells. The cells were co-transfected with 10 ng pFC-PKA with either wt LG or m3 together with the β-gal expression vector and incubated overnight, before harvesting. Due to the standard errors there appears to be no statistically significance between LG/β-gal/Basic and LG/β-gal/PKA and no statistically significance between m3/β-gal/Basic and m3/β-gal/PKA. Standard errors are shown. Results of one experiment are shown above. This experiment was repeated five times in triplicate and the same trend was observed for all experiments. ** represents a P value of less than 0.01 (P<0.01), relative to the control.

Over-expression of the PKA protein resulted in an increase in the activity of wt LG promoter of approximately 1.5 fold over cells that were not co-transfected with PKA (Figure 26) indicating that PKA is involved in positive regulation of the GnRHR promoter. When the mutated site 3 construct was transfected into LβT2 cells and over-expressed with the PKA protein a slight difference was observed. Wt LG together with PKA resulted in a ~2-fold increase over basal levels (see bars 1 and 2) of the GnRHR promoter in LβT2 cells. The mutated site 3 construct resulted in a ~ 1.2 fold increase over basal levels (compare bars 3 and 4) of the wt LG promoter in LβT2 cells. This suggests that site 3 is partially involved in the PKA response although it is not absolutely required for the response. There is no evidence that PKA was produced in transfected cells although it could have been confirmed by performing Western blots.

3.6.2 Is the AP-1 site involved in the PKA response?

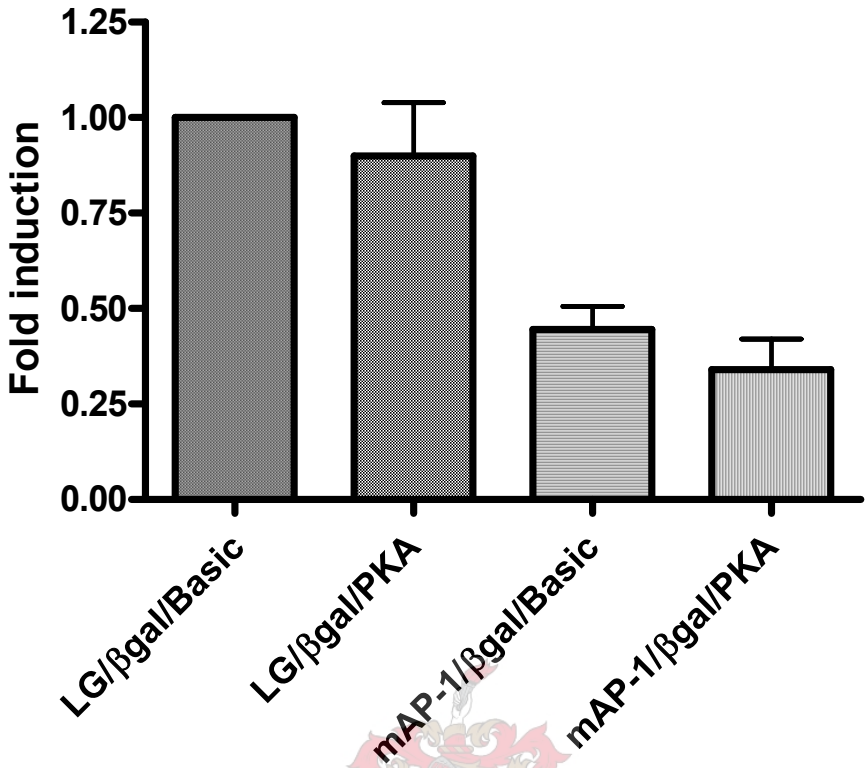


Figure 27: The effect of PKA on the GnRHR promoter in LβT2 cells. The cells were co-transfected with 10 ng pFC-PKA with either wt LG or the mutated AP-1 site construct together with the β-gal expression vector and incubated overnight, before harvesting. Due to the standard errors there appears to be no statistically significance between LG/β-gal/Basic and LG/β-gal/PKA and no statistically significance between mAP-1/β-gal/Basic and mAP-1/β-gal/PKA. The graph is a result of combined data. This experiment was repeated four times in duplicate.

According to figure 27, there appears to be a slight decrease in the PKA response when compared to the wt LG, unlike the increase observed in figure 26. The mutant AP-1 basal responsiveness has the same trend as seen in figure 22 showing a ~50% decrease in the basal transcriptional levels. From figure 27 it is difficult to conclude if the AP-1 site is involved in the PKA response. The small changes observed were not statistically significantly different to each other, most likely since the small effect was within the range of the experimental error and variable. It is thus possible that in this system the AP-1 site is involved in mediating a small PKA response, as found by Lin *et al.* (1999) [34] in GGH_3 cells where GnRH activation of the rat GnRHR promoter occurred via cAMP and protein kinase A pathways. However due to the high degree of variability in the response in the current system, the results were inconclusive.

3.7 The role of site 3 and or AP-1 sites in the SF-1 response

An investigation to determine whether over-expressing the SF-1 protein regulates the GnRHR promoter gene in LβT2 cells was carried out together with the role of site 3 and the AP-1 site. The SF-1 protein was over-expressed in LβT2 cells using transient transfections of an expression vector for SF-1. Secondly, functional studies were carried out to provide functional evidence for the role of the SF-1 protein, since previous experiments suggested that site 3 and the AP-1 site were both involved in regulating basal levels of expression of the GnRHR promoter gene in LβT2 cells (See figure 21 and 22 respectively). The effect of the SF-1 protein on the AP-1 site was carried out in order to determine if there was any form of cross-talk between the AP-1 site and the SF-1 site, which was speculated by some researchers [87].

3.7.1 Is site 3 involved in the SF-1 response?

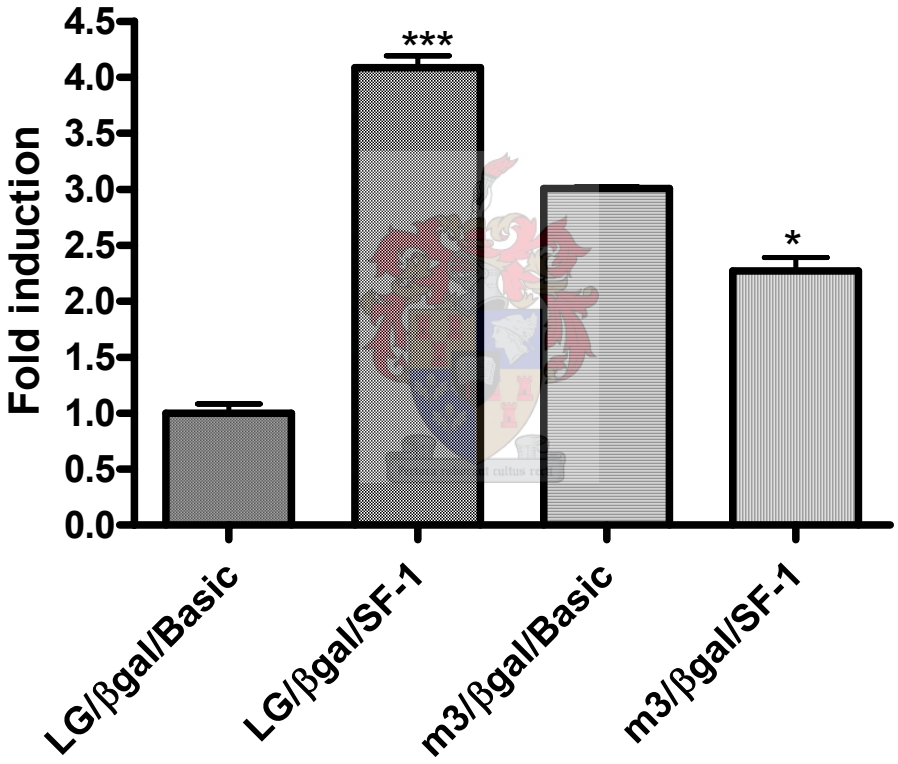


Figure 28: Representative graph showing the effect of over-expressed pCMV-SF-1 on wt LG and m3. 20 ng pCMV-SF-1 was co-transfected with 100 ng wt LG and 10 ng pSV-β-gal expression construct into LβT2 cells and cells were incubated overnight. The graph above is from one representative experiment. Standard errors are shown. This experiment was repeated three times in triplicate and the same trend was observed for all experiments. The * and *** represents a P value of 0.05 ($P<0.05$) and 0.001 ($P<0.001$), relative to the control, respectively.

As shown in figure 28, the presence of 20 ng pCMV-SF-1 increased pLG activity by 4-fold (compare bars 1 and 2). This was expected as the GnRHR promoter contains 2 known SF-1-like sites and a third

putative SF-1 site (site 3), which was being investigated. When site 3 was mutated a 3-fold increase in basal expression relative to the pLG construct was observed, similar to the result shown in figure 21. Unlike the result with the wt construct when site 3 was mutated in the presence of the SF-1 expression vector resulted in a decrease in the expression levels as compared to mutated site 3 construct in the absence of the SF-1 expression vector (compare bars 3 with 4). The loss of the positive response to over-expression of the SF-1 protein seen with the wt construct, after mutation of site 3, strongly suggest that site 3 is required for mediating a positive response to SF-1 protein.

Other researchers found that when site 2 (-244/-236 NRS) was mutated in αT3-1 cells a complete loss in the SF-1-induced increased in expression levels seen with the wt construct was observed [80]. However, mutation of site 1 (-15/-7 NRS) did not result in a loss of the increase in expression levels obtained in αT3-1 cells in the presence of over-expressed with SF-1 [80]. Therefore, it can be said that the SF-1-like site 2 is an important site for mediating stimulatory effects of SF-1 protein on the expression levels of the GnRHR promoter gene in αT3-1 cells. A comparison between site 3 and site 2 shows that the differences between these two sites are 2 base pairs (7/9 bases are identical), suggesting that the same factors bind at these two sites.

Thus, site 2 is required for mediating the response to SF-1 protein in αT3-1 cells whereas site 3 is required for mediating the response to SF-1 protein in LβT2 cells. These different results in the two cell lines could be due to cell specific differences in the signalling pathways. Alternatively, there is a possibility that both these sites are required to be intact in both cell lines although this has not yet been tested. Further studies could lead us to a deeper understanding about the role of these two sites in two cell lines.

3.7.2 Is the AP-1 site involved in the SF-1 response?

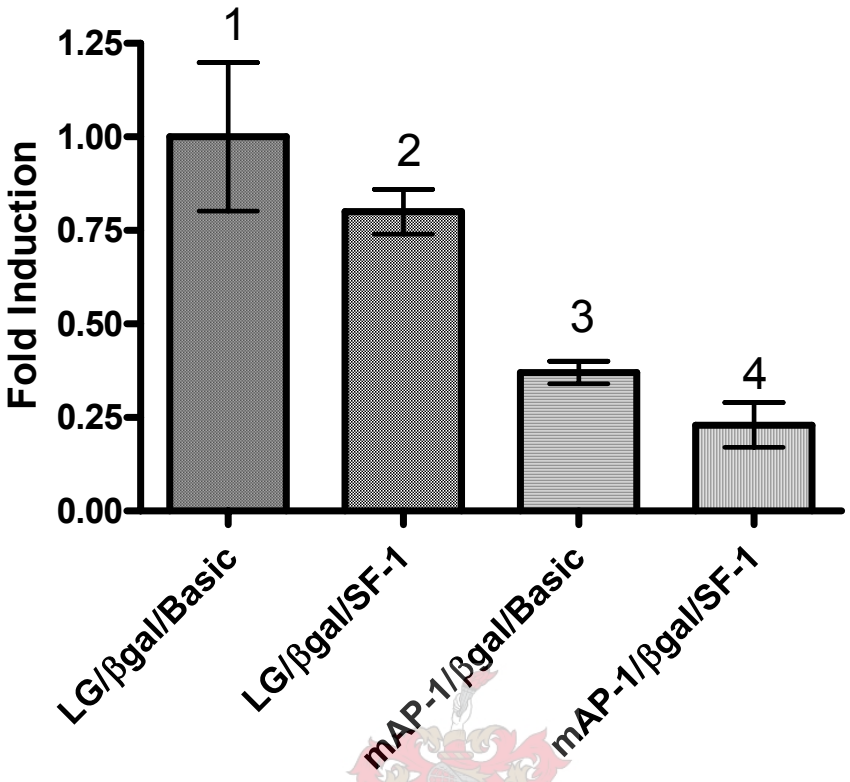


Figure 29: Representative graph showing the effect of over-expressed SF-1 on wt LG and on the mAP-1 site. 20 ng pCMV-SF-1 was co-transfected with 100 ng wt LG and 10 ng pSV-β-gal expression construct into LβT2 cells and the cells were incubated overnight. The graph above is a result of one representative experiment. Standard errors are shown. This experiment was repeated three times in triplicate and the same trend was observed for all experiments. There was no statistical significance when comparing bars 1 and 2 and comparing bars 3 and 4.

Figure 29, showed no increase in the expression levels between wt LG in the presence of the pCMV-SF-1 expression vector as compared to in the absence of the pCMV-SF-1 expression vector, unlike the effects seen in figure 28. Thus it was not possible to determine whether the AP-1 site was involved in mediating the SF-1 response due to the high degree of variability in the response of the cells.

3.8 The GnRHR promoter is regulated by dexamethasone

An important question that was asked was whether dexamethasone (dex) regulates the GnRHR promoter gene in LβT2 cells and if so, which sites are important for this regulation in LβT2 cells. Researchers such as Maya Nunez *et al.* (2003) [69] (in GGH_3 cells) and Turgeon *et al*. (1996) [43] (LβT2 cells) found that the GnRHR promoter was responsive to the synthetic glucocorticoid, dex in the respective cell lines mentioned. In the present study, LβT2 cells were transfected with pLG and incubated overnight. The next day the cells were stimulated with different concentrations of dex (10,

100 and 1000 nM) for varying times (30 minutes, 4, 8 and 16 hours). The cells were then harvested and assayed for expression levels (see figure 30).

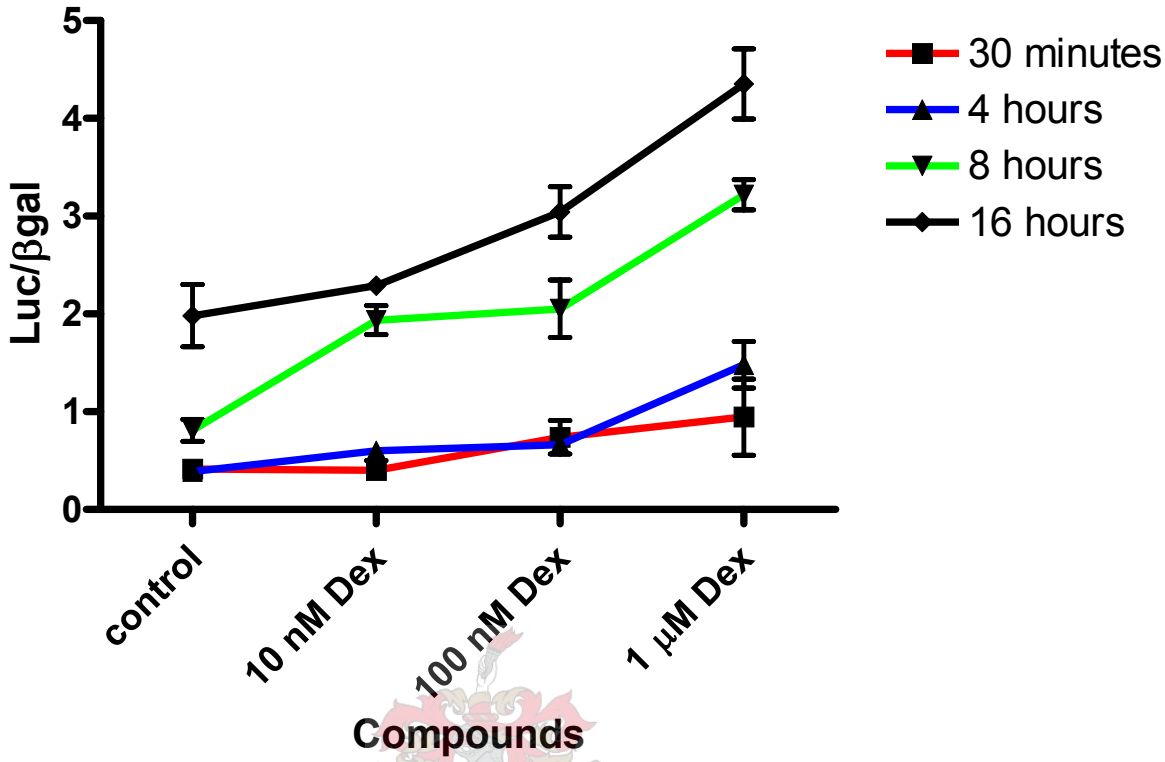


Figure 30: A time and dose response of wild type GnRHR reporter gene to dex. LβT2 cells were transfected with 100 ng wt LG together with a 10 ng pSV-β-gal expression vector and incubated with different concentrations of dex (10 nM, 100 nM, 1 μM, respectively) and varying time points (30 minutes, 4 hours, 8 hours and 16 hours). The control for this experiment was 0.1% ethanol for each time point. Cells were then harvested depending on their time point. The graph is a result of combined data. Standard error bars are shown and this experiment was carried out four times in triplicate.

Figure 30 shows that the GnRHR promoter-reporter construct in LβT2 cells responds positively to dex. Between all time points the basal levels of the wt LG increases, most likely due to cell growth effects. At 30 minutes and at 4 hours there appears to be a slight response at the higher concentration of dex. At 8 hours there is an increase with 10 and 100 nM and a further increase when 1 μM dex is added. The greatest increase was observed at 16 hours with 1 μM dex, which is consistent with results obtained in GGH_3 cells [69]. These researchers also found that if GGH_3 cells were further stimulated for up to 24 hours that there is a decrease in the response [69]. It would be interesting to see if they were stimulated for longer than 16 hours, which will be the subject of future studies.

3.8.1 Is site 3 involved in the dexamethasone response of the GnRHR promoter?

An investigation was performed to determine which *cis*-elements on the GnRHR promoter are important for the positive regulation found when stimulated with dex. Constructs used for these experiments contained either the wt LG or those in which site 3 or the AP-1 site on the GnRHR promoter were

mutated. The constructs were transfected into LβT2 cells together with the pSV-β-gal expression vector and the cells were incubated overnight. The next day the cells were stimulated with 1μM dex for 16 hours before being harvested and assayed.

As one can see from figure 31 when cells were stimulated with dex it resulted in a 1.5-fold increase in reporter expression over uninduced wt LG, which is similar to results shown in figure 30. Mutation of site 3 (plasmid m3) resulted in a ~6 fold increase of basal levels over the wt LG control. The increase shown in figure 31 is greater than that shown in figure 21, reflecting the high degree of variability of the response of the cells. When m3 was stimulated with 1 μM dex a loss of the dex response was observed when compared with the increase seen with wt LG when stimulated with dex. This result suggests that site 3 is required for the dex response.

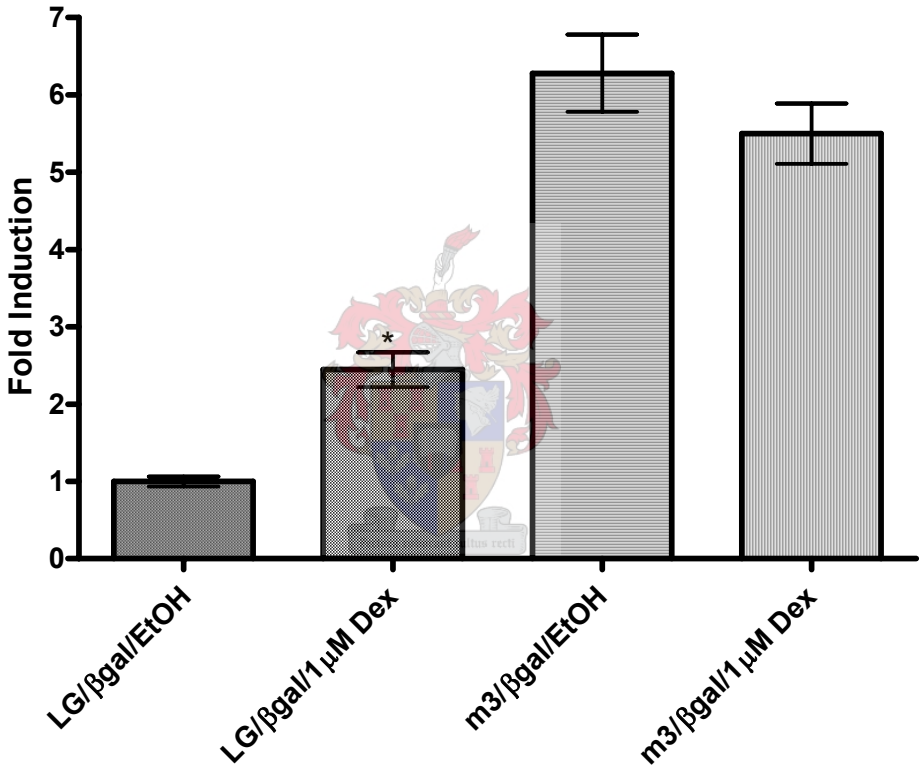


Figure 31: A representative graph showing the role of site 3 in the response to dex on the GnRHR promoter in LβT2 cells. 100 ng wt LG or 100 ng m3 were transfected into LβT2 cells together with 10 ng of β-gal expression vector and cells were stimulated either with or without 1 μM dex and incubated for 16 hours, before harvesting. The graph shows the result of one representative experiment. Standard errors are shown and this experiment was carried out three times in triplicate. The * represents a P value of less than 0.05 ($P < 0.05$), relative to the control (bar 1).

3.8.2 Is the AP-1 site involved in the dexamethasone response of the GnRHR promoter?

The AP-1 site was investigated to determine if it had a significant role in the positive regulation of the GnRHR promoter by dex.

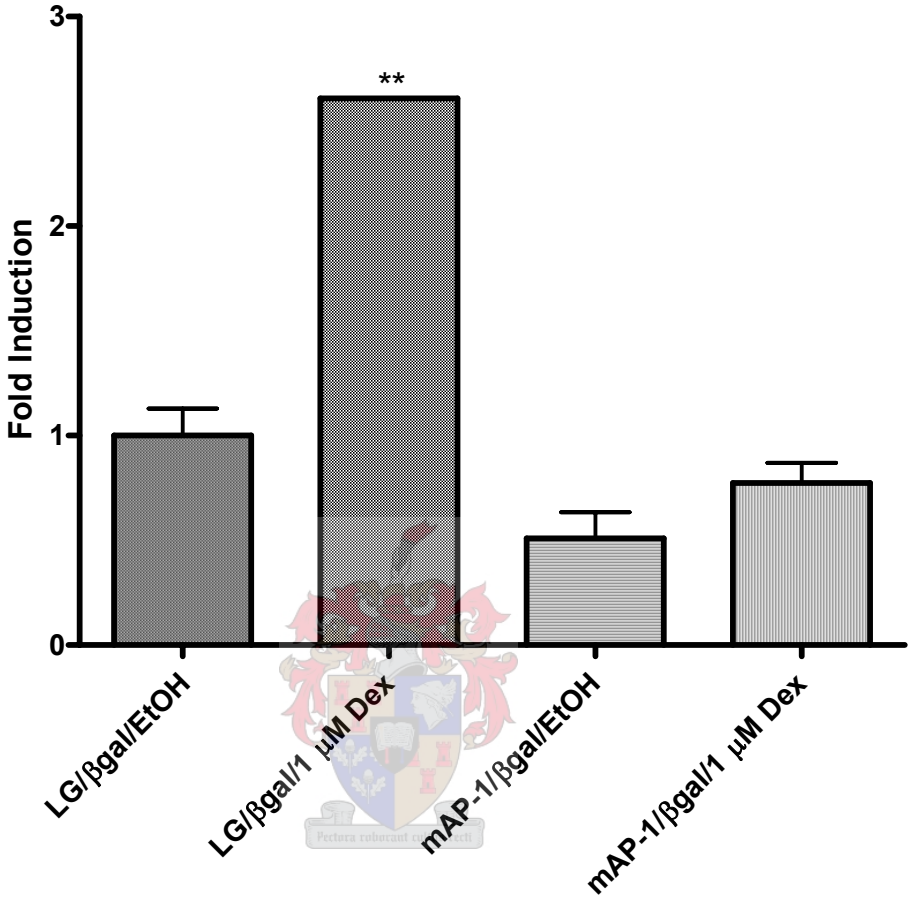


Figure 32: A representative graph showing the role of the AP-1 site in the response to dex on the GnRHR promoter in LβT2 cells. 100 ng wt LG or 100 ng mAP-1 were transfected into LβT2 cells together with 10 ng of β-gal expression vector and cells were stimulated either with or without 1 μM dex and incubated for 16 hours, before harvesting. The graph shows the result of one representative experiment. Standard errors are shown and this experiment was carried out three times in triplicate. The ** represents a P value of less than 0.01 (P< 0.01), relative to the control.

As shown in figure 32, wt LG expression was stimulated with 1 μM Dex showing a 2.5-fold increase. When the AP-1 site was mutated it caused a 50% decrease in basal transcriptional activity, which is in line with what was shown in figure 22. Mutation of the AP-1 site resulted in complete loss of the dex response when stimulated with 1 μM dex. These results show that the AP-1 site is required for the positive regulation of the GnRHR promoter by dex in the LβT2 cells. Maya-Nunez *et al.* (2003) [69] found the same results in GGH_3 cells as we found in LβT2 cells suggesting that the effect seen with dex is not cell specific.

It can be said from these studies that site 3 and the AP-1 site play a significant role in mediating the dexamethasone response of the GnRHR promoter in LβT2 cells. The result for AP-1 has not been previously found by others in this specific cell line while no other investigators have previously investigated the role of site 3.

Conclusion

The mouse GnRH receptor (mGnRHR) promoter contains two established SF-1 DNA binding sites located at positions –244/-236 and –15/-7 relative to the translational start site and a third putative SF-1 binding site (site 3) with unknown function located at position –304/-296. In this study the role of the SF-1 site 3 and the AP-1 site (-336/-330) in the transcriptional regulation of the mGnRHR promoter in LβT2 cells was investigated.

An overview of the finding results are given below in the table.

	wt LG	**Site 3**	**AP-1 site**
GnRH	Yes	No	No
Overexpressed SF-1	Yes	Yes	ND
Overexpressed PKA	Yes	Partial	ND
Dexamethasone	Yes	Yes	Yes

Table 2: An overview to the results obtained with regards to the mGnRHR promoter activity being increased in LβT2 cells. “Yes” indicates where *cis* elements are involved. “No” indicates where *cis* elements are not involved. Partial indicates where *cis* elements are partially involved. “ND” indicates not determined and requires further experimentation.

Earlier studies performed on site 1 of the mGnRHR promoter found that site 1 was not important in the mediation of basal levels [80] in αT3-1 cells. However a positive role for site 2 of the mGnRHR promoter in mediating basal expression levels in αT3-1 cells was previously established [14, 80]. Experiments carried out with site 3 on the mGnRHR promoter showed that site 3 is involved in basal regulation. Findings were that when site 3 was mutated it resulted in a 1.5 fold increase over wt LG, which suggests that site 3 acts as an inhibitory element in LβT2 cells (see figure 33).

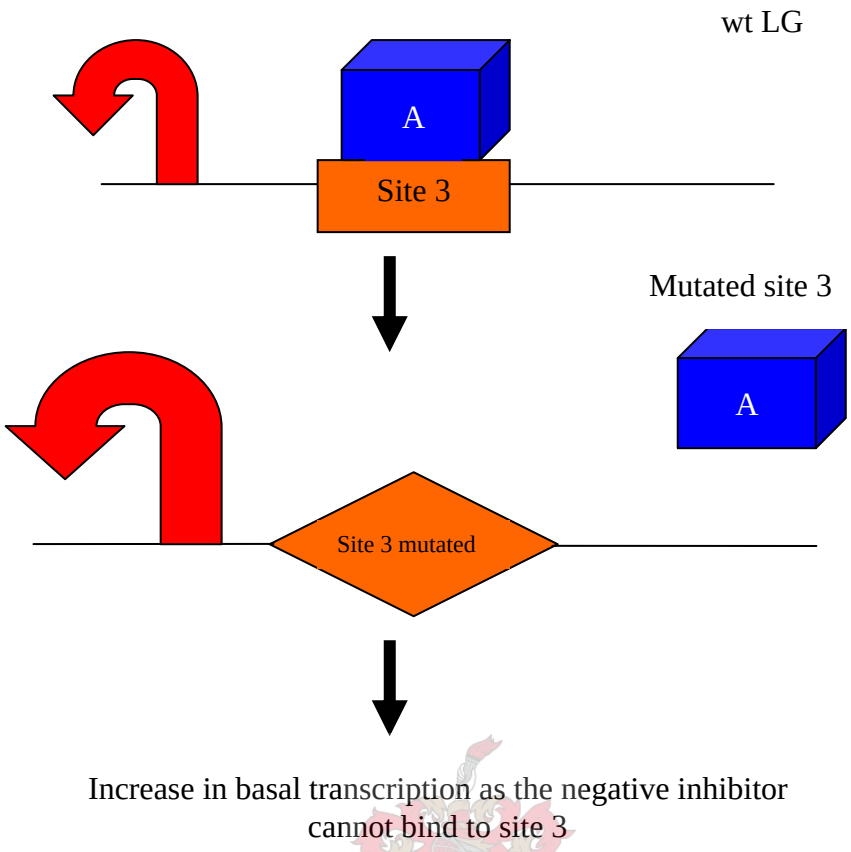


Figure 33: A schematic diagram showing an increase in basal levels when site 3 was mutated on the GnRHR promoter in LβT2 cells. A = negative inhibitor. This diagram is supported by figure 21.

It was also evident that the AP-1 site is involved in the basal levels of mGnRHR promoter activity in LβT2 cells. When the AP-1 site was mutated it resulted in a 50% decrease in basal transcriptional levels suggesting that the AP-1 site acts as a stimulatory element in LβT2 cells, possibly via binding of a stimulatory protein. The findings for the AP-1 site are in line with other researchers in GGH_3 cells [69] and in αT3-1 cells [14, 18]. One can conclude from this that the AP-1 site on the mGnRHR promoter is important in basal transcriptional levels in several pituitary cell lines.

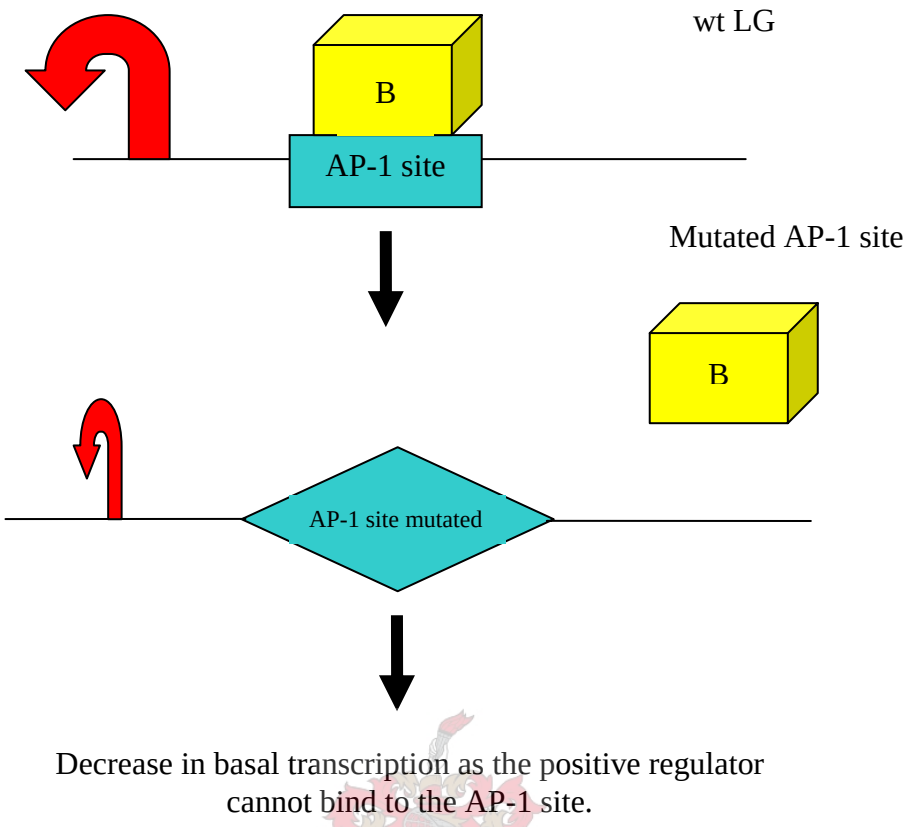


Figure 34: A schematic diagram showing a decrease in basal levels when the AP-1 site is mutated on the GnRHR promoter in LβT2 cells. B = positive regulator. This diagram is supported by figure 22.

The role of the AP-1 site and site 3 was investigated with regards to the upregulation of the wild type mGnRHR promoter when stimulated with GnRH in LβT2 cells. It has been previously shown that the AP-1 site is required to mediate a GnRH-mediated upregulation in αT3-1 cells [18]. In contrast to these researchers the findings in this study were that the AP-1 site is not involved in the GnRH response of the GnRHR promoter in LβT2 cells. Site 3 was not involved either in the GnRH response in LβT2 cells. This could be due to cell specific differences between LβT2 cells and αT3-1 cells. For example it is possible that a GnRH response is mediated by different *cis* elements in LβT2 cells. The type of stimulation (continuous versus pulsatile) in LβT2 cells could also affect the type of response that is observed. There is currently much controversy as to which type of stimulation gives the optimum results. In this study continuous administration of GnRH was performed, which is suggested by Kaiser *et al.* (1997) [1] to result in a maximum of 2-fold increase as opposed to much higher values in primary pituitary cells. From the literature it is known that GnRH does play a role in the transcription of the mGnRHR promoter leading to PKA activating CREB and that GnRH activates the PKA pathway via the G_s pathway in LβT2 cells, therefore one could speculate that GnRH acts via CREB.

In this study the PKA response was difficult to study due to various factors affecting the experiment. These reasons are as follows: a high degree of variability in the responses, time of day the experiment was carried out, the passage number of the cells, contamination of media used, or the temperature of media used. In these studies it was not possible to show whether the AP-1 or the SF-1 site on the mGnRHR promoter was involved in the PKA response in LβT2 cells, since only a small non-reproducible effect was observed with PKA.

Site 3 of the mGnRHR promoter gene binds the SF-1 protein from nuclear extracts of LβT2 cells *in vitro*. When site 3 was mutated a complete loss in the SF-1 response was observed in LβT2 cells. This is interesting as no experiments have been performed on this site before and the role of this site was thus previously unknown. A role for site 3 in mediating the increase in transcription by SF-1 in line with the roles of two other established SF-1 sites [80].

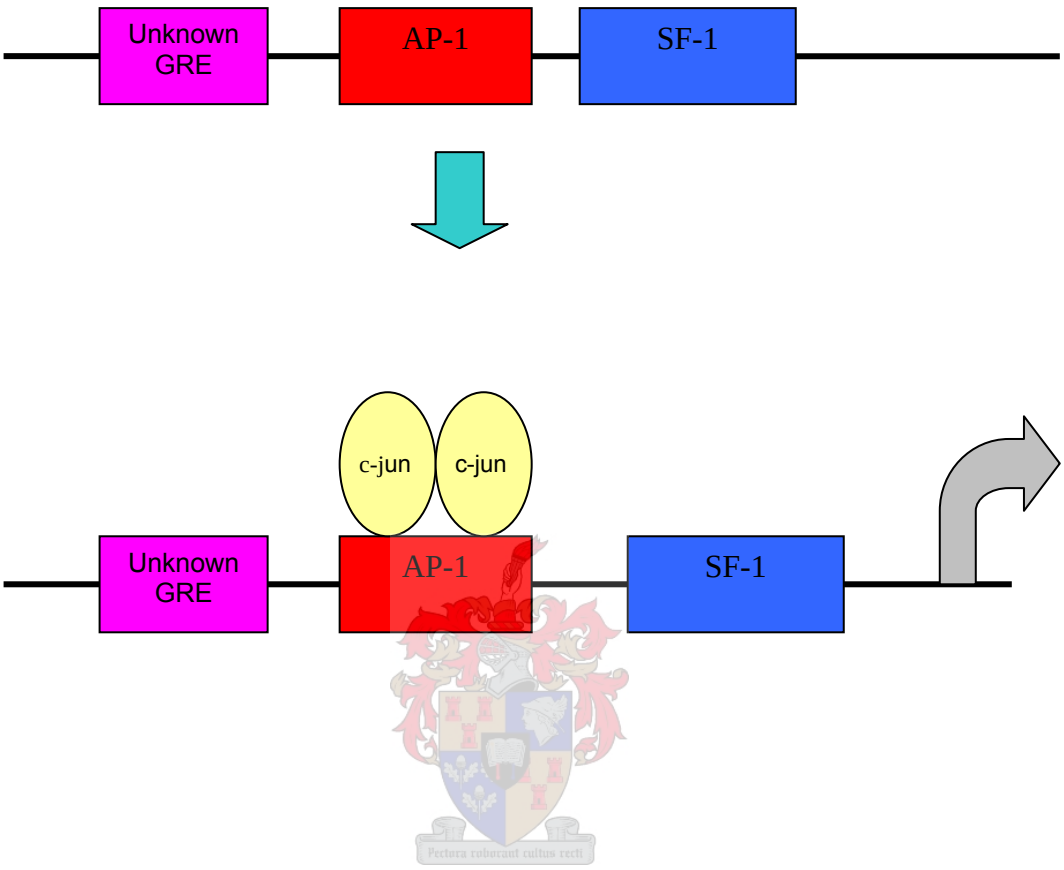
Lastly, whether the mGnRHR promoter was responsive to a synthetic glucocorticoid, dexamethasone in LβT2 cells was investigated. It was shown that dex up-regulates the mGnRHR promoter in LβT2 cells and that this response requires site 3 as well as the AP-1 site, since the mutation of either one of these sites resulted in a loss of the dex response. It is known from experiments above that in the absence of dex or GnRH that something binds to the AP-1 site *in vitro* and it has been shown that the proteins that do bind to the AP-1 site do not include c-Fos. At present it is not known what binds to the AP-1 site in the presence of GnRH and Dex.

A possible model is described in figure 35 showing the two different pathways that can be involved in the stimulation of the GnRHR promoter when dexamethasone is present.

No Dex

A

mGnRHR
promoter



B **With Dex there are 2 scenarios**

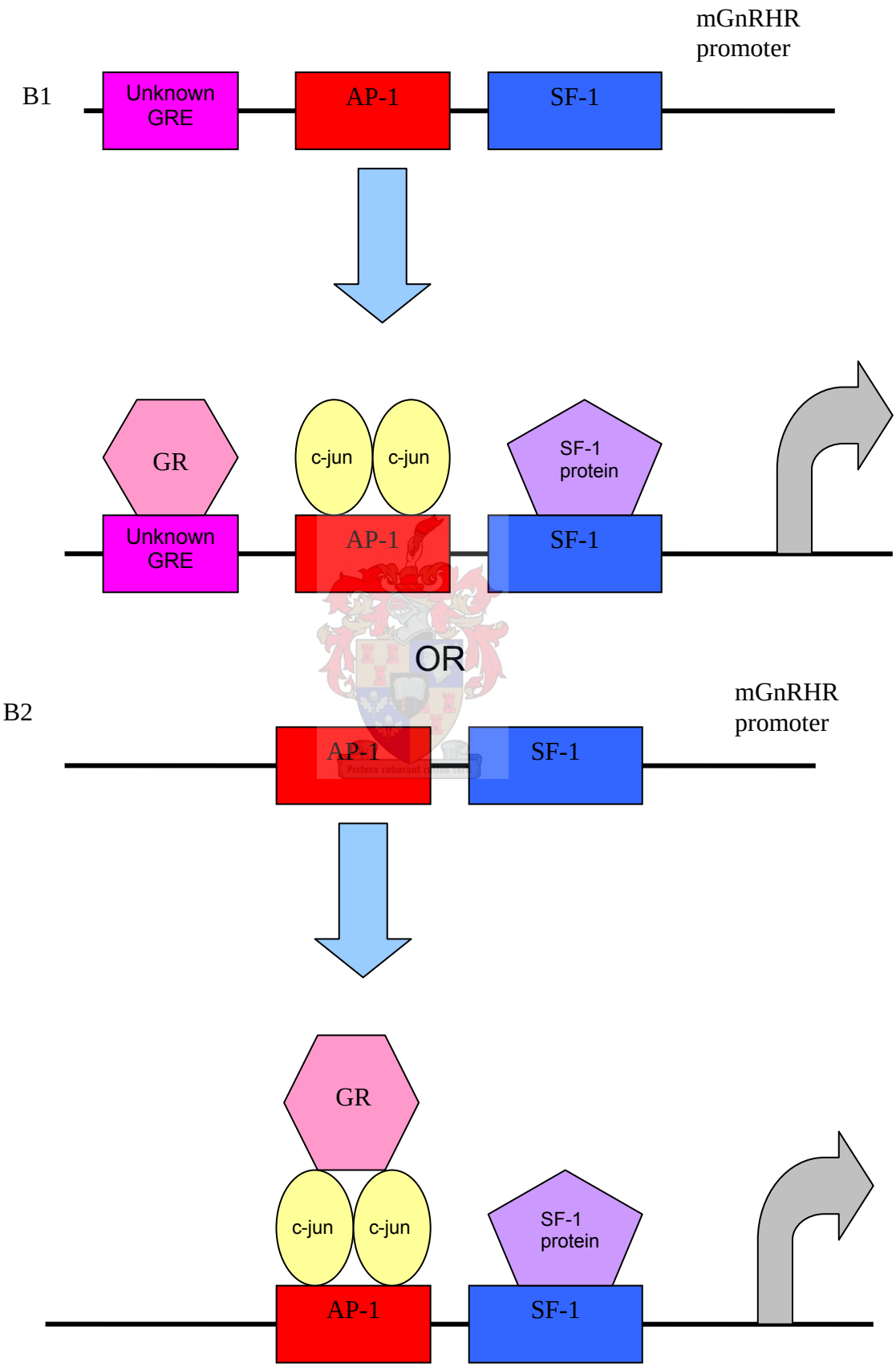


Figure 35: Schematic representation of the effect of the presence or absence of dexamethasone on the AP-1 site and site 3 on the mGnRHR promoter in LβT2 cells. **A** = no dex. When no dex is present c-jun homodimers bind to the AP-1 site resulting in transcription of GnRHR promoter.
B = Two scenarios for the presence of dex. B(1) In the presence of dex the GR binds to an "unknown" GRE in close proximity to the AP-1 site, the c-jun homodimer binds to the AP-1 site and the SF-1 protein binds to the SF-1 site. All three proteins interact with DNA causing a looping out effect. A greater increase in transcription of the mGnRHR promoter is observed as compared to when no dex is present. B(2) The same effect is observed for B(2) as in B(1), when dex is present, but in this model GR does not bind to the DNA, it directly interacts and binds with the AP-1 c-jun homodimer resulting in a greater transcriptional increase of the mGnRHR promoter as compared to when no dex was present. Diagram by Fernandes (2006).

From experiments shown earlier (see figure 10) the data suggests that under basal conditions c-Fos does not bind to the AP-1 site leading to the speculation that c-Jun binds as a homodimer to the AP-1 site on the mGnRHR promoter in LβT2 cells. Research performed by others suggested that when GnRH was present an increase in the c-fos protein was observed, increasing the possibility that the AP-1 protein would bind to the AP-1 site as a heterodimer. Another possibility is that on the mGnRHR promoter there is a non-classical GRE site ~21bp from the AP-1 site. Literature suggests that if a GRE and AP-1 binding site overlap and the AP-1 site is occupied by c-jun homodimer and the GRE is occupied by the liganded GR then it results in an increase in transcription of the mGnRHR promoter or alternatively a decrease in transcription of the mGnRHR promoter if both c-Jun and c-Fos is present. In order for transactivation or transrepression to occur by binding of the GR and the AP-1 proteins to the GRE-AP-1 site the GRE-AP-1 site must be 26bp or greater apart to transactivate or 14-18bp apart to transactivate or transrepress [67] in the presence of dex.

The results obtained in this study would be consistent with a model whereby a non-classical GRE occurs in the murine GnRHR promoter which partially overlaps with the AP-1 site. Therefore, when the AP-1 site is mutated a loss in transcription of the mGnRHR promoter is observed when dex is present.

A possible interpretation of the result for site 3 where a decrease in transcription was observed when site 3 was mutated, transfected into LβT2 cells and stimulated with dex could possibly be that when LβT2 cells were stimulated with dex an increase in SF-1 protein occurred, which cannot bind to the SF-1 site (since site 3 is mutated) resulting in a decrease in transcription of the mGnRHR promoter, although no studies have been carried out on this speculation. Another possible speculation is that site 3 is in close proximity to the AP-1 site and that cross-talk could occur between these two sites. For a full transcriptional response of the mGnRHR promoter to occur when stimulated with dex both sites are required to be present on the mGnRHR promoter resulting in the increase in transcription of the mGnRHR promoter. If one of the sites were mutated the transcriptional activity of the mGnRHR promoter was compromised. The mutation of both site 3 and the AP-1 site on the mGnRHR promoter has not yet been carried out in the presence of dex and it would be interesting to see if a response even

if minimal (due to other SF-1 sites being present) is still observed or if a complete loss in the dex response would occur.

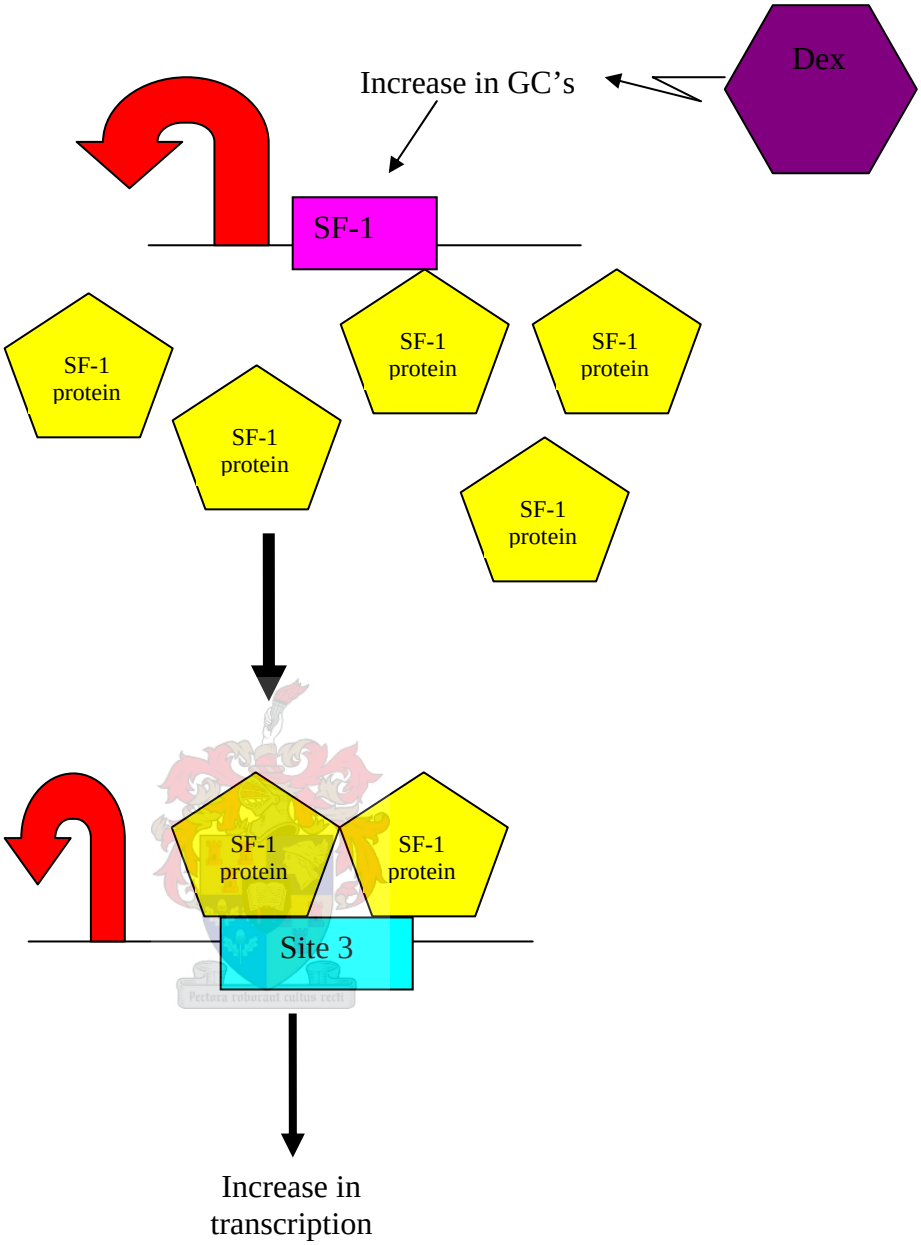


Figure 36: Schematic diagram showing a possible model of how the glucocorticoid receptor responds to dex causing increases in SF-1 protein levels which then results in increased binding of SF-1 protein to site 3 in the murine GnRHR promoter and increased transcription thereof. This diagram is supported by figure 31.

The experimentations carried out in this thesis were designed to gain insight into the relationship between stress and reproduction. The findings were very interesting in that an increase in transcription of the mGnRHR promoter in LβT2 cells was seen after 16 hours of stimulation with dex, while no increase was observed at 30 min or at 4 hr, similar to the results of Maya-Nunez *et al.* (2003) [69]. Maya-Nunez *et al.* (2003) [69] also found that when cells were stimulated for longer than 16 hours with dex that

a decrease in transcription of the GnRHR promoter in GGH_3 cells was observed. It is well established that chronic stress results in a decrease in reproduction. However, the effects of short term stress on reproduction are not well understood. The findings in this study suggest that when woman are mildly stressed an increase in reproduction can occur, that may be mediated via an increase in GnRHR levels, with a resulting increase in sensitivity to GnRH in the pituitary. These findings support the literature mentioned earlier where it was suggested that under some conditions that stress can have a positive effect on reproduction, and suggest a possible mechanism for this effect [69].

Future studies

Results presented in this thesis have raised many interesting questions for future studies. The following list described below gives examples of some of the questions that have been raised to be investigated for future studies.

- Whether the proteins that bind to site 3 and the AP-1 site are required to interact for different responses requires further experimentation.
- In particular, the effects of dexamethasone on site 3 need to be further investigated to determine whether the response requires cross-talk between proteins binding to site 3 and the AP-1 site. This could be further investigated by performing simultaneous mutations of both sites.
- Further experiments are required to determine whether indirect effects play a role in the dex response such as whether dex results in an increase in SF-1 protein expression levels.
- Whether c-Jun binds to the AP-1 site as a homodimer under certain circumstances, while c-Fos and c-Jun bind as a heterodimer under others needs to be investigated.
- The details of the kinase pathway involved in regulating the AP-1 site and site 3 need to be further investigated to understand whether the PKC pathway is largely responsible in the regulation of these sites or whether other kinase pathways also play a role such as the PKA pathway.
- It would also be interesting to investigate whether any other *cis* elements mediate the dex response such as possible non-classical GREs.

Addendum A

6.1 Preparation of the Solutions

6.1.1 TE buffer (pH 8)

10 mM Tris.HCl (pH8)

1 mM EDTA (pH8)

6.1.2 Luria-Bertani (LB) Medium

10 g Tryptone

5 g yeast extract

10 g Sodium Chloride

Mixture is made up to 1 L with de-ionized water

Autoclave to sterilize.

6.1.3 SOC Medium

20 g Tryptone

5 g Yeast tryptone

0.5 g Sodium Choloride

Mixture is made up to 1L with deionized water.

10ml of a 250 mM solution KCl was added to the above. The pH was adjusted to 7.0 with NaOH. The volume was then adjusted to 1 L. The mixture was then sterilized by autoclaving. Before use 5 ml sterile 2M $MgCL_2$ and 20 ml sterile 1 M solution of glucose was added to the mixture. The mixture was then aliquoted and stored at -20°C.

6.1.4 50x TAE

242 g Tris Base

57.1 ml Glacial acetic acid

100 ml 0.5 M EDTA (pH 8)

Make up to 1 L using de-ionized water.

6.2 Gel mobility shift assays

6.2.1 5% acrylamide gel mixture

6.6 ml Acrylamide solution (29:1 molar ratio of acrylamide to bisacrylamide.)

32.5 ml Sterile deionized Water

400 µl 50 x TAE

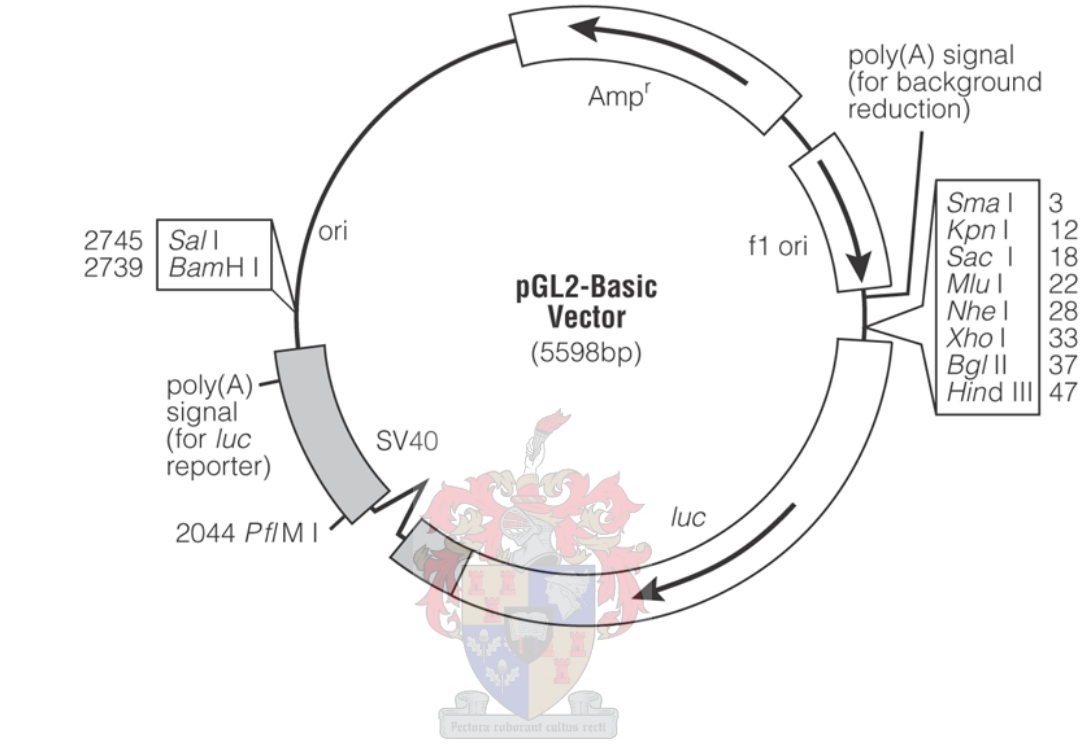
400 µl 10% (w/v) Ammonium Persulphate (APS) (BDH AnalaR)

70 µl TEMED (Merck)

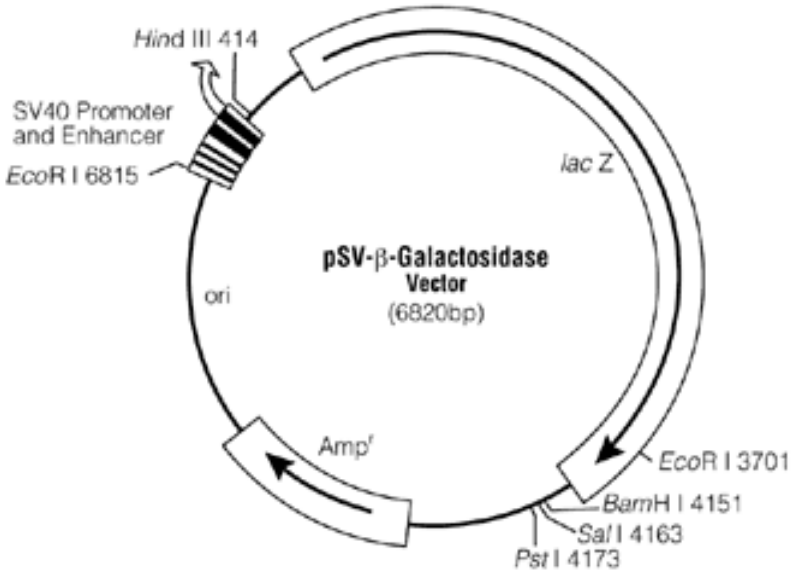
ADDENDUM B

PLASMID MAPS

B1: pGL2-Basic vector map and multiple cloning site sequence



B2: pSV-β-Galactosidase expression vector



B3: pFC-PKA:

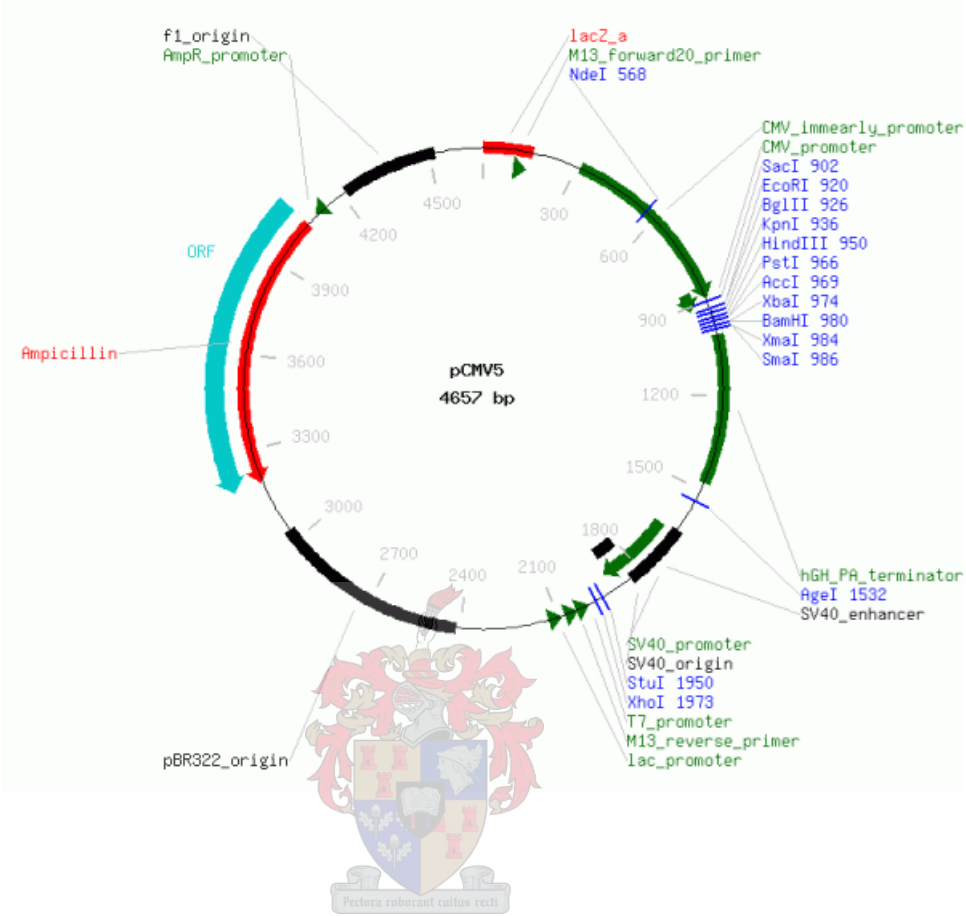
No map was available for this construct. Technical information from Stratagene was as follows:

Size: 7180 bp

Restriction pattern: *Hind*III, *Xba*I, *Xho*I: 480 bp, 500 bp, 1200 bp, 5000 bp

B4: Other vector backbones used for expression constructs

E5.1 pCMV5 (i.e. SF-1)



References

[1] Kaiser UB, Conn M and Chin WW (1997). Studies of Gonadotropin-Releasing Hormone (GnRH) action using GnRH Receptor-Expressing pituitary cell lines. *Endocrinology Reviews*; **18:**46-69.

[2] Norwitz ER, Jeong KH and Chin WW (1999). Molecular mechanisms of gonadotropin-releasing hormone receptor gene regulation. *Journal of the Society for Gynecologic Investigation;* **6:**169-178.

[3] Hapgood JP, Sadie H, van Biljon W, and Ronacher K (2005). Regulation of expression of mammalian GnRH receptor genes. *Journal of Neuroendocrinology.* **17:**619-638(20).

[4] Jiang Z, Gibson JP, Archibald AL, Haley CS (2001). The porcine gonadotropin-releasing hormone receptor gene (GNRHR): genomic organization, polymorphisms, and association with the number of corpora luteal. *Genome;* **44:**7-12.

[5] Granger A, Ngo-Muller V, Bleux C, Guigon C, Pincas H, Magre S, Daegelen D, Tixier-Vidal A, Counis R, Laverriere JN (2004). The Promoter of the Rat Gonadotropin-Releasing Hormone Receptor Gene Directs the Expression of the Human Placental Alkaline Phosphatase Reporter Gene in Gonadotrope Cells in the Anterior Pituitary land as well as in Multiple Extra-pituitary Tissues. *Endocrinology*; **145:**983-993.

[6] Zapatero-Caballero H, Sanchez-Franco F, Guerra-Perez N, Fernandez-Mendez C, Fernandez-Vazquez G (2003). Gonadotropin-Releasing Hormone Receptor Gene Expression during Pubertal Development of Male Rats. *Biology of Reproduction;* **68:**1764-1770.

[7] Bauer-Dantoin AC, Hollenberg AN, Jameson JL (1993). Dynamic regulation of gonadotropin-releasing hormone receptor mRNA levels in the anterior pituitary gland during the rat estrous cycle. *Endocrinology;* **133:**1911-1914.

[8] Millar RP, Lu Z, Pawson AJ, Flanagan CA, Morgan K, and Maudsley SR (2004). Gonadotropin-Releasing Hormone Receptors. *Endocrine Reviews;* **25:**235-275.

[9] Bedecarrats GY and Kaiser UB (2003). Differential regulation of Gonadotropin sub-unit gene promoter activity by pulsatile gonadotropin-Releasing Hormone (GnRH) in Perifused LβT2 cells: Role of GnRH Receptor concentration. *Endocrinology;* **144:**1802-1811.

[10] Norwitz ER, Jeong KH, and Chin WW (1999). Molecular mechanisms of Gonadotropin-releasing hormone receptor gene regulation. *Society for Gynecologic Investigation;* **6:**169-178.

[11] Yasin M, Dalkin AC, Haisenleder DJ, Kerrigan JR, Marshall JC (1995). Gonadotropin-releasing hormone (GnRH) pulse pattern regulates GnRH receptor gene expression: augmentation by estradiol. *Endocrinology;* **36:**1559-1564.

[12] Albarracin CT, Kaiser UB and Chin WW (1994). Isolation and characterization of the 5'-flanking region of the mouse Gonadotropin-Releasing hormone receptor gene. *Endocrinology;* **135:**2300-2306.

[13] Norwitz ER, Cardona GR, Jeong KH, Chin WW (1999). Identification and Characterization of the Gonadotropin-releasing Hormone Response Elements in the Mouse Gonadotropin-releasing Hormone Receptor Gene. Journal *of Biological Chemistry;* **274:**867-880.

[14] Duval DL, Nelson SE, and Clay CM (1997). The tripartite basal enhancer of the gonadotropin-releasing hormone receptor gene promoter regulates cell-specific expression through a novel GnRH receptor activating sequence. *Molecular Endocrinology;* **11:**1814-1821.

[15] Reinhart J, Xiao S, Arora KK, and Catt KJ (1997). Structural organization and characterization of the promoter of the rat gonadotropin-releasing hormone receptor gene. *Molecular and Cellular Endocrinology*; **130:**1-12

[16] Pincas H, Forrai Z, Chauvin S, Laverriere JN, and Counis R (1998). Multiple elements in the distal part of the 1.2 kb 5'-flanking region of the rat GnRH receptor gene regulate gonadotrope-specific expression conferred by proximal domain. *Molecular and Cellular Endocrinology*; **144:**95-108.

[17] Maya-Nunez G and Conn PM (1999). Transcriptional regulation of the gonadotropin-releasing hormone receptor gene is mediated in part by a putative repressor element and by the cyclic adenosine 3`, 5`-monophosphate response element. *Endocrinology;* **140:**3452-3458.

[18] White BR, Duval DL, Mulvaney JM, Roberson MS and Clay CM (1999). Homologous regulation of the Gonadotropin-Releasing hormone receptor gene is partially mediated by protein kinase C activation of an activator protein element. *Molecular Endocrinology*; **13:**566-577.

[19] Val P, Lefrancois-Martinez A, Veyssiere G, and Martinez A (2003). SF-1 a key player in the development and differentiation of steroidogenic tissues. *Nuclear Receptor*; **1:**1-23.

[20] Honda S, Morohashi K, Nomura M, Takeya H, Kitajima M and Omura T (1993). Ad4BP regulating steroidogenic P-450 gene is a member of steroid hormone receptor superfamily. *Journal of biological chemistry;* **268:**7494-7502.

[21] Fan W, Yanase T, Wu Y, Sawate H, Saitoh M, Oba K, Nomura M, Okabe T, Goto K, Yanagisawa J, Kato S, Ryoichi T and Nawata H (2004). Protein Kinase A potentiates adrenal 4 binding protein/steroidogenic factor 1 transactivation by reintegrating the subcellular dynamic interactions of the nuclear receptor with its cofactors, general control nonderepressed-5/transformation/ transcription domain-associated protein, and suppressor, dosage-sensitive sex reversal-1: a Laser confocal imaging study in living KGN cells. *Molecular Endocrinology;* **18:**127-141.

[22] Morohashi K, Honda S, Inomata Y, Handa H and Omura T (1992). A common transacting factor, Ad4-binding protein, to the promoters of steroidogenic P-450s. *Journal of Biological Chemistry;* **267:**17913-17919.

[23] Parker KL and Schimmer BP (1997). Steroidogenic Factor-1: A key determinant of endocrine development and function. *Endocrine Reviews;* **18:**361-377.

[24] Achermann JC, Ito GOM, Orun UA, Harmanci K, Gurakan B and Jameson JL (2002). Gonadal determination and adrenal development are regulated by the orphan nuclear receptor steroidogenic factor-1, in a dose dependent manner. *The Journal of clinical Endocrinology and Metabolism*; **87:**1829-1833.

[25] Luo X, Ikeda Y and Parker KL (1995). The cell specific nuclear receptor Steroidogenic factor-1 plays multiple roles in reproductive function. *Philosophical Transactions of the Royal Society of London*; **350:**279-283.

[26] Hammer GD, Krylova I, Zhang Y, Darimont BD, Simpson K, Weigel NL and Ingraham HA (1999). Phosphorylation of the nuclear receptor SF-1 modulates cofactor recruitment: Integration of hormone signalling in reproduction and stress. *Molecular Endocrinology;* **3:**521-526.

[27] Ngan ESW, Cheng PKW, Leung PCK and Chow BKC (1999). Steroidogenic Factor-1 interacts with Gonadotrope specific element within the first exon of the human Gonadotropin-Releasing Hormone Receptor gene to mediate Gonadotrope-specific expression. *Endocrinology;* **140:**2452-2462.

[28] Shapiro DB, Pappalardo A, White BA and Peluso JJ (1996). Steroidogenic factor-1 as a positive regulator of rat granulose cell differentiation and a negative regulator of mitosis. *Endocrinology;* **137:**1187-1195.

[29] Zhang P and Mellon SH (1996). The orphan nuclear receptor Steroidogenic factor-1 regulates the cyclic adenosine 3`, 5`-monophosphate-mediated transcriptional activation of rat cytochrome P450c17 (17α-Hydroxylase/c17-20 lyase). *Molecular Endocrinology*; **10:**147-158.

[30] Young M and McPhaul MJ (1998). A steroidogenic factor-1 binding site and cyclic adenosine 3`, 5`-monophosphate response element like elements are required for the activity of the rat aromatase promoter in rat Leydig tumor cell lines. *Endocrinology;* **139:**5082-5093.

[31] Michael MD, Kilgore MQ, Morohashi K, and Simpson ER (1995). Ad4BP/SF-1 regulates cyclic AMP-induced transcription from the proximal promoter of the human aromatase P450 (CYP19) gene in the ovary. *Journal of Biological Chemistry*; **270:**13561-13566.

[32] Lin X and Conn PM (1998). Transcriptional activation of GnRHR gene by GnRH and cyclic adenosine monophosphate. *Endocrinology;* **139:**3896-3902.

[33] Naor Z, Benard O and Seger R (2000). Activation of MAPK cascades by G-protein coupled receptors: The case of Gonadotropin-Releasing Hormone Receptor. *Trends in Endocrinology and Molecular Biology;* **11:**91-99.

[34] Lin X and Conn PM (1999). Transcriptional activation of Gonadotropin-releasing hormone (GnRH) Receptor Gene by GnRH: Involvement of multiple signal transduction pathways. *Endocrinology;* **140:**358-364.

[35] Windle JJ, Weiner RI, and Mellon PL (1990). Cell lines of the pituitary gonadotrope lineage derived by targeted oncogenesis in transgenic mice. *Molecular Endocrinology;* **4:**597-603.

[36] McArdle CA, Schomerus E, Groner I and Poch A (1992). Estradiol regulates gonadotropin-releasing hormone receptor number, growth and inositol phosphate production in αT3-1 cells. *Molecular and Cellular Endocrinology;* **87:**95-103.

[37] Thomas P, Mellon PL, Turgeon JL and Waring DW (1996). The LβT2 clonal gonadotrope: A model for single cell studies of endocrine cell secretion. *Endocrinology;* **137:**2979-2989.

[38] Pincas H, Laverriere JN, Counis R (2001). Pituitary Adenylate Cyclase-activating Polypeptide and Cyclic Adenosine 3',5'-Monophosphate Stimulate the Promoter Activity of the Rat Gonadotropin-releasing Hormone Receptor Gene via a Bipartite Response Element in Gonadotrope-derived Cells. *Journal of Biological Chemistry;* **276:**23562-23571.

[39] Tsutsumi M, Laws SC, Rodic V, Sealfon SC (1995). Translational regulation of the gonadotropin-releasing hormone receptor in alpha T3-1 cells. *Endocrinology;* **136:**1128-1136.

[40] Mason DR, Arora KK, Mertz LM, Catt KJ (1994). Homologous down-regulation of gonadotropin-releasing hormone receptor sites and messenger ribonucleic acid transcripts in alpha T3-1 cells. *Endocrinology*; **135:**1165-1170.

[41] Kam KY, Jeong KH, Norwitz ER, Jorgensen EM, Kaiser UB (2005). Oct-1 and Nuclear Factor Y Bind to the SURG-1 Element to Direct Basal and Gonadotropin-Releasing Hormone (GnRH)-Stimulated Mouse GnRH Receptor Gene Transcription. *Molecular Endocrinology*; **19:**148-162.

[42] Ellsworth BS, White BR, Burns AT, Cherrington BD, Otis AM and Clay CM (2003). c-Jun N-treminal kinase activation of activator protein-1 underlies homologous regulation of the gonadotropin-releasing hormone receptor gene in T3-1 cells. *Endocrinology;* **144:**839-849.

[43] Turgeon JL, Kimura Y, Waring DW, and Mellon PL (1996). Steroid and Pulsatile Gonadotropin-Releasing Hormone (GnRH) regulation of Luteinizing hormone and GnRH receptor in a novel gonadotrope cell line. *Molecular Endocrinology*; **10:**439-450.

[44] Cheng KW and Leung PCK (2001). Human gonadotropin releasing hormone receptor gene transcription: up-regulation by 3`5`-cyclic adenosine monophosphate/protein kinase A pathway. *Molecular and Cellular Endocrinology*; **181:**15-26.

[45] Kaiser UB, Jakubowiak A, Steinberger A, Chin WW (1993). Regulation of rat pituitary gonadotropin-releasing hormone receptor mRNA levels in vivo and in vitro. *Endocrinology;* **133:**931-934.

[46] Haisenleder DJ, Cox ME, Parsons SJ, Marshall JC (1998). Gonadotropin-Releasing Hormone Pulses Are Required to Maintain Activation of Mitogen-Activated Protein Kinase: Role in Stimulation of Gonadotrope Gene Expression. *Endocrinology;* **139:**3104-3111.

[47] Borgeat P, Chavancy G, Dupont A, Labrie F, Arimura A, Schally AV (1972). Stimulation of adenosine 3':5'-cyclic monophosphate accumulation in anterior pituitary gland in vitro by synthetic luteinizing hormone-releasing hormone. *Proceedings of the National Academy of Science;* **69:**2677-2681.

[48] Stanislaus D, Ponder S, Ji TH and Conn M (1998). Gonadotropin-Releasing Hormone Receptor couples to multiple G proteins in rat gonadotrophs and in GGH3: Evidence from palmitoylation and overexpression of G proteins. *Biology of Reproduction;* **59:**579-586.

[49] Reiss N, Llevi LN, Shacham S and Haris D (1997). Mechanism of mitogen-activated protein kinase activation by Gonadotropin-Releasing Hormone in the pituitary αT3-1 cell lines: Differential roles of calcium and protein kinase C. *Endocrinology;* **138:**1673-1682.

[50] De Bosscher K, Vanden Berghe W, and Haegeman G (2001). Glucocorticoid Repression of AP-1 is not mediated by competition for nuclear co-activators. *Molecular Endocrinology;* **15:**219-227.

[51] Smoak KA and Cidlowski JA (2004). Mechanisms of glucocorticoid receptor signalling during inflammation. *Mechanisms of Ageing and Development*; **125:**697-706.

[52] Ferin M (1999). Clinical Review 105: Stress and the reproductive cycle. The *Journal of Clinical Endocrinology and Metabolism;* **84:**1768-1774.

[53] Reifenstein EC (1946). Psychogenic or "Hypothalamic" amenorrhea. *Medical Clinics North America;* **30:**1103-1114.

[54] Berga SL, Mortola JF, Girton L, Suh B, Laughlin G, Pham P, and Yen SSC (1989). Neuroendocrine aberrations in women with functional hypothalamic amenorrhea. *Journal of Clinical Endocrinology and Metabolism*; **68:**301-308.

[55] Chandran UR, Attardi B, Friedman R, Dong KW, Roberts JL, and DeFranco DB (1994). Glucocorticoid receptor-mediated repression of gonadotropin-releasing hormone promoter activity in GT1 hypothalamic cell lines. *Endocrinology*; **134:**1467-1474.

[56] Dubey AK, and Plant TM (1985). A suppression of gonadotropin secretion by cortisol in castrated male rhesus monkeys mediated by the interruption of hypothalamic gonadotropin-releasing hormone release. *Biology ofl Reproduction*; **33:**423-431.

[57] Baldwin DM, Srivastava PS and Krummen LA (1991). Differential actions of corticosterone on luteinizing hormone and follicle-stimulating hormone biosynthesis and release in cultured rat anterior pituitary cells: interaction with estradiol. *Biology of Reproduction*; **44:**1040.

[58] Tilbrook AJ, Turner AI, Clarke IJ (2000). Effects of stress on reproduction in non-rodent mammals: the role of glucocorticoids and sex differences. *Reviews in Reproduction*; **5:**105-113.

[59] Robinson-Rechavi M, Carpentier AS, Duffraisse M, Laudet V (2001). How many nuclear hormone receptors are there in the human genome? *Trends in Genetics;* **17:**554-556.

[60] Beato M, Herrlich P, Schutz G (1995). Steroid hormone receptors: many actors in search of a plot. *Cell;* **83:**851-857.

[61] Kumar R and Thompson EB (2003). Transactivation functions of the N-terminal domains of nuclear hormone receptors: protein folding and coactivator interactions. *Molecular Endocrinology*; **17:**1-10.

[62] Pujols L, Mullol J, Terrego A, Picado C (2004). Glucocorticoid receptors in human airways. *Allergy;*. **59:**1042-1052.

[63] Webster JC, and Cidlowski JA (1999). Mechanisms of Glucocorticoid-receptor-mediated repression of gene expression. *Trends in Endocrinology and Molecular Biology*; **10:**396-402.

[64] Strahl BD, Haung HG, Pederson NR, Wu JC, Ghosh BR and Miller WL (1997). Two proximal activating protein-1 binding sites are sufficient to stimulate transcription of the ovine Follicle Stimulating Hormone-gene. *Endocrinology;* **138:**2621-2631.

[65] Drouin J, Maira M and Philips A (1998). Novel mechanism of action for Nurr-77 and antagonism by glucocorticoids: a convergent mechanism for CRH action and glucocorticoid repression of POMC gene transcription. *Journal of Steroid Biochemistry and Molecular Biology*; **65:**59-63.

[66] Schule R (1990). Functional antagonism between oncoprotein c-Jun and the glucocorticoid receptor. *Cell;* **62:**1217-1226.

[67] Pearce D, Matsui W, Miner JN, and Yamamoto KR (1998). Glucocorticoid receptor transcriptional activity determined by spacing of receptor and non-receptor DNA sites. *Journal of Biological Chemistry*; **273:**30081-30085.

[68] Karin M, Liu Z and Zandi E (1997). AP-1 function and regulation. *Current Opinion in Cell Biology;* **9:**240-246.

[69] Maya-Nunez G and Conn PM (2003). Transcriptional regulation of the GnRH receptor gene by glucocorticoids. *Molecular and Cellular Endocrinology;* **200:**89-98.

[70] Cheng KWA, Ngan ESW, Kang SK, Chow BKC and Leung PCK (2000). Transcriptional down-regulation of human gonadotropin-releasing hormone (GnRH) receptor gene by GnRH: role of Protein Kinase C and Activating Protein 1. *Endocrinology;* **141:**3611-3622.

[71] Campion CE, Turzillo AM, Clay CM (1996). The gene encoding the ovine gonadotropin-releasing hormone (GnRH) receptor: cloning and initial characterization. *Gene;* **170:**277-280.

[72] Crowder ME, Nett TM (1984). Pituitary content of gonadotropins and receptors for gonadotropin-releasing hormone (GnRH) and hypothalamic content of GnRH during the periovulatory period of the ewe. *Endocrinology;* **114:**234-239.

[73] Turzillo AM, Nolan TE, Nett TM (1998). Regulation of Gonadotropin-Releasing Hormone (GnRH) Receptor Gene Expression in Sheep: Interaction of GnRH and Estradiol. *Endocrinology;*. **139:**4890-4894.

[74] Looper ML, Vizcarra JA,Wettemann RP, Malayer JR, Braden TD, Geisert RD, Morgan GL (2003). Influence of estradiol, progesterone, and nutrition on concentrations of gonadotropins and GnRH receptors, and abundance of mRNA for GnRH receptors and gonadotropin subunits in pituitary glands of beef cows. *Journal of Animal Science;* **81:**269-278.

[75] Savoy-Moore RT, Schwartz NB, Duncan JA, Marshall JC (1980). Pituitary gonadotropin-releasing hormone receptors during the rat estrous cycle. *Science;* **209:**942-944.

[76] Turzillo AM, Campion CE, Clay CM, Nett TM (1994). Regulation of gonadotropin-releasing hormone (GnRH) receptor messenger ribonucleic acid and GnRH receptors during the early preovulatory period in the ewe. *Endocrinology;* **135:**1353-1358.

[77] Batra SK, Miller WL (1985). Progesterone decreases the responsiveness of ovine pituitary cultures to luteinizing hormone-releasing hormone. *Endocrinology;* **117:**1436-1440.

[78] Sakurai H, Adams BM, Adams TE (1997). Concentration of GnRH receptor and GnRH receptor mRNA in pituitary tissue of orchidectomized sheep: effect of oestradiol, progesterone, and progesterone withdrawal, *Journal of Endocrinology;* **152:**91-98.

[79] Cheng KW, Cheng CK, Leung PCK (2001). Differential Role of PR-A and -B Isoforms in Transcription Regulation of Human GnRH Receptor Gene. *Molecular Endocrinology;* **15:**2078-2092.

[80] Sadie H, Styger G and Hapgood J (2003). Expression of the mouse gonadotropin-releasing hormone receptor gene in αT3-1 Gonadotrope cells is stimulated by cyclic 3`, 5`-Adenosine monophosphate and protein kinase A, and is modulated by Steroidogenic Factor-1 and Nur77. *Endocrinology;* **144:**1958-1971.

[81] Horn F, Windle JJ, Barnhart KM and Mellon PL (1992). Tissue specific gene expression in the pituitary: the glycoprotein hormone alpha-subunit gene is regulated by a gonadotrope-specific protein. *Molecular and Cellular Biology*. **12:**2143-2153.

[82] Sambrook J, Fritsch EF, and Maniates T (1989). Molecular cloning: A laboratory manual, 2nd edition, Cold Spring Harbor, New York.

[83] Wumbach E, Yuen T, Ebersole BJ and Sealfon SC (2001). Gonadotropin-releasing hormone receptor-coupled gene network organization. *Journal of Biological Chemistry;* **276:**47195-201.

[85] Styger G (2001). The role of Steroidogenic Factor-1 (SF-1) in Transcriptional regulation of the Gonadotropin releasing hormone (GnRH) Receptor gene. M.Sc. Thesis Department of Biochemistry, University of Stellenbosch, Stellenbosch.

[86] Young LS, Naik SI, and Clayton RN (1984). Adenosine 3', 5'-monophosphate derivatives increase gonadotropin-releasing hormone receptors in cultured pituitary cells. *Endocrinology;* **114:**2114-2122.

[87] Li LA, Lala D, and Chung B (1998). Function of Steroidogenic factor 1 (SF1) ligand-binding domain in gene activation and interaction with AP1. *Biochemical and Biophysical Research Communications*; **250**:318-320.